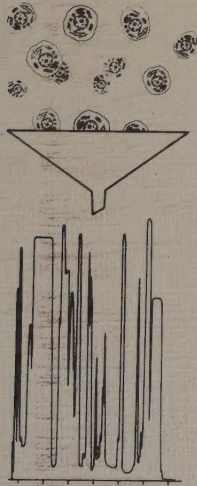


LUND 1983

NUCLEOTIDE METABOLISM
IN VARIOUS CELL SYSTEMS IN VIVO AND IN VITRO
STUDIED BY ISOTACHOPHORESIS

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This thesis is a summary of the following papers (referred to by their Roman numerals) and some additional unpublished observations

- I A method for measuring nucleotides in the liver of rat with isotachopheresis. *Analytical Biochemistry* 109, 239-246, 1980.
- II Decreased UDP-glucuronic acid in rat liver after ether narcosis. Together with Daniel Stråth. *FEBS Letters* 124, 39-42, 1981.
- III The effect of 5-fluoroorotic acid on the early labelling of nucleotides and RNA in whole liver and of RNA in sub-nuclear fractions in rats given ^3H -orotic acid. Together with Unne Stenram. *Analytical and preparative isotachopheresis*. Ed. C J Holloway, *Analytical Chemistry Symposia Series Vol. 7*, Elsevier Scientific Publishing Company, Amsterdam (1983), in press.
- IV Isotachopheretic determination of the base composition in rat liver RNA with and without a column coupling system adapted to a tachophor capillary. *Analytical and preparative isotachopheresis*. Ed. C J Holloway, *Analytical Chemistry Symposia Series Vol. 7*, Elsevier Scientific Publishing Company, Amsterdam (1983), in press.
- V Effects of γ -radiation and hyperthermia on DNA repair synthesis and the level of NAD^+ in cultured human mononuclear leukocytes. Together with Göran G Jonsson and Ronald W Pero. Resubmitted to *Radiation Research*.
- VI Equilibration of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and UDP-N-acetylhexosamine with labelled precursors in cultured fibroblasts. Together with Bengt Särnstrand and Anders Malmström. Submitted to *Experimental Cell Research*.



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INTRODUCTION

Our laboratory has studied the structure and function of the RNA-containing cell organelles and the metabolism of RNA in rat liver for several years. The effects of protein deficiency and of cytostatic drugs, e.g. fluoro compounds, are our main interest. Such experiments affect those nucleotides that are precursors of RNA. Capillary isotachopheresis was considered as a suitable method for analysis of these nucleotides.

This study demonstrates the application of the isotachopheretic method to the complex extract of liver acid-soluble compounds. In addition we dealt with problems relating to tissue sampling, extraction and large differences in concentrations. A column coupling system adapted to the Tachophor capillary is described. With i.a. this method the effects of fluoroorotic acid on the labelling pattern of nucleotides and RNA after administration of ^3H -orotic acid were studied in the liver of rat.

The results of these studies lead up to collaboration with other research groups working on nucleotide problems in other cell systems. In human mononuclear leukocytes the method is used for measuring ATP, energy charge and NAD^+ , to get a better understanding of some of the metabolic events involved in DNA repair after γ -radiation and/or heat treatment. In fetal human skin fibroblasts in culture, nucleotides and the equilibration of the precursors of glycosaminoglycans are measured after administration of labelled ^{35}S -sulfate and ^3H -glucosamine.

GENERAL METHODS

Animals and liver sampling

Male Wistar rats (150-200 g) from Møllegaard (Denmark) or from our own strain were fed a 25% casein diet for 7 days (1) and given water ad libitum. The experiments were performed between 8 and 10 a.m.

Liver sampling with divinylether as the anaesthetic agent is described in paper II. An improvement of that method is the use of a pair of tongs precooled in liquid nitrogen for in situ sampling.

Extraction of liver nucleotides

The experimental model with improvements, used in this study, has been described in papers I, III, V and VI. The present mode of extraction is summarized here.

The clamped rat liver is pulverized under liquid N_2 . The powder is weighed in a cryostat (Linde, GFR) at $-20^{\circ}C$ so that a suitable amount, $50-100\text{ mg} \pm 0.25\text{ mg}$, can be extracted in 7 parts of a solution containing 0.7 M perchloric acid ($HClO_4$), 30% (v/v) methanol and 0.5 mM nicotinic acid. The nucleotides are extracted under continuous shaking for 20 min and centrifuged for 1 min at 30000g and $-20^{\circ}C$ (setting temperature) in a Sorvall (USA) RC2-B centrifuge with an SS-34 rotor. In the cryostat 200 μ l of the supernatant is transferred to 30 μ l of a mixture containing 16 μ l 7 M KOH, 10 μ l 0.4 M triethanolamine, buffered with HCl to pH 7.2, and 4 μ l thymol blue (3 mg/10 ml 8 mM KOH). With a 10 μ l syringe, 2M KOH is added and, after the colour change, pH is carefully adjusted to pH 7.0-7.2 as determined by an indicator paper (pH-range 6.4-8.0, Merck, GFR). The neutralized extract is centrifuged for 5 min at 30000g and $-20^{\circ}C$ in order to obtain a higher precipitation of potassium perchlorate. The supernatant is stored at $-80^{\circ}C$ for later use.

Mononuclear leukocytes

The preparation of human venous blood is described in paper V. The procedure followed in these experiments was always the same. In the morning

fresh blood was collected, the leukocytes were prepared and the experiments were performed immediately. In all experiments two samples were taken before any treatment and those were referred to as controls. The other samples were put in waterbaths at different temperatures (37°C - 45°C) for 45 min, and then placed on ice, where one group was given radiation and the other was untreated. After this procedure the samples were incubated for various times at 37°C . After centrifugation the cell pellets were stored at -80°C and extracted the following morning. The extraction procedure was the same as for liver, but due to the small extraction volumes, specially made micro-homogenizers with pestels were used.

Fibroblasts

In paper VI the treatment of the cells and the preparation of nucleotides from embryonic human fibroblasts in culture is described. In the present experiments the cell pellets are extracted in the cryostat at -20°C as are the mononuclear leukocytes. The only difference is that the fibroblasts are sonicated in a Bransonic (USA) 32 sonifier bath after the addition of extraction medium.

Other preparation methods

Nucleotides, RNA and DNA were prepared according to Munro and Fleck (2). DNA was determined by different techniques. For liver the pellet remaining after RNA hydrolysis was used for analysis of DNA by UV-absorbance according to Scott et al (3) as modified by Hinrichs et al (4). DNA in mononuclear leukocytes was determined by a modification of Burton's diphenylamine method (5) described by Pero et al (6). In fibroblasts the DNA was determined on the pellet remaining after extraction of nucleotides by two fluorescence methods using mitramycin (7) or bisbenzimidazole (8).

The preparation of sub-nuclear morphological fractions of liver is described in paper III. Other methods used for studying different subgroups of RNA are the phenol extraction of nuclear RNA (see papers III and IV), gel electrophoresis (see paper III) and total RNA hydrolysis for base analysis with isotachophoresis (see paper IV).

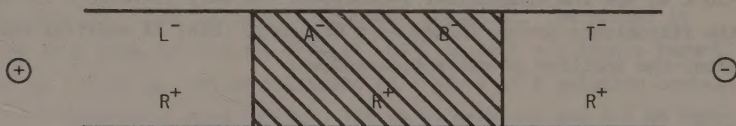
ISOTACHOPHORESIS

Theory

Isotachophoresis means migration of ions in an electric field with all ions moving at the same velocity. This migration takes place in a system consisting of;

- I. A leading ion with the same sign as and a higher effective mobility than the sample ions to be separated.
- II. A terminating ion with the same sign and a lower effective mobility than the sample ions to be separated.
- III. A polarity that directs the movement of ions to be detected towards the leading electrode (fig. 1).

Fig. 1. Illustration of isotachophoretic separation



L = leading ion in leading electrolyte

T = terminating ion in terminating electrolyte

R = buffering counter ion

A, B = sample ions

When sample ions are injected into a system for the separation of negative ions between the leading and the terminating zone and when electric current is applied, the ions to be separated will start to migrate towards the anode according to:

$$v = m \times E \quad (1)$$

v = velocity

m = effective mobility

E = electrical field strength

In the beginning the different sample ions will migrate with different velocities because the electrical field strength is equal in the mixed sample zone and the effective mobilities are different (see formula 1). At steady state the ions are arranged as zones in order of effective mobility and are forced to move in direct contact with each other and with equal velocity assuming a homogeneous current density. As a consequence isotachopheresis has a zone sharpening effect which counteracts diffusion and thus results in high resolution.

$$\frac{C_A}{C_B} = \frac{m_A}{m_A + m_R} \times \frac{m_B + m_R}{m_B} \quad (2)$$

$$C_A = C_L \times \text{const.} \quad (3)$$

C = concentration

m = effective mobility

A, B, R and L = ions (see fig. 1)

Kohlrausch (9) described the so-called regulating function (see formula 2) which means that the concentration of a sample zone will adapt itself to the concentration of the preceding zone. Consequently the concentration of a sample zone is directly proportional to the concentration of the leading ion (see formula 3). Through this function it can be seen that there is a concentration-effect in isotachopheresis. For example, a diluted sample will be concentrated until its concentration is correlated to that of the leading ion. The reverse obtains for a sample with a concentration higher than that of the leading ion.

Practical aspects and results

The isotachopheresis was performed on an LKB 2127 Tachophor (LKB, Sweden), as described in paper I. A Tarkan W+W recorder 900 (W+W, Switzerland) and an LDC 304:50 computing integrator (LDC, England) were used for registration and measurement of the UV-signals.

The electrolyte system described in paper I is still used though with a few improvements. The stock solution of HCl is now made from 0.1 M Titrisol (Merck, GFR). β -alanine (Sigma Chemicals, USA) is recrystallized

in methanol. Hydroxypropylmethylcellulose (HPMC) (Dow Chemicals Co, USA) is stirred in water at a concentration of 1% (w/v) for 24 h at +4°C. The solution is then filtered under N₂-pressure with a maximum of 2 kp/cm² in a Millipore stainless steel pressure vessel having an AP 20 filter and an AP 25 prefilter in the filterholder (Millipore Corp, USA). Not even this prefilter can prevent clogging and the filters have to be changed every 250-350 ml. After a second run through the filters the solution is ion-exchanged in an Amberlite monobed resin MB-1 (BDH Chemicals Ltd, England). This so-called 1% stock solution of HPMC is stored at +4°C.

The operation of the tachophor is described in paper I. Especially important is to use an effect lower than 2.5 W to prevent air bubble formation. Analytical isotachophoresis at our laboratory is today mainly run in a 43 cm teflon tube and with UV-detection but with no need for thermal detection. This implies that the teflon tube (Habia, Sweden) is easy to exchange and can be repaired at a low cost. Woledge and Reilly (10) used the thermal detector for automatic triggering of the integrator and the recorder paper drive. The automatic triggering system at our laboratory is connected to the UV-signal and switches on the recorder paper drive after an increase of UV-absorption by 2-3%. This means that naturally occurring oxalic acid or UTP starts the recorder paper drive in our system. The integrator is started manually after the 0-100% UV-absorption setting, which is made 5-10 min before registration.

Preparative isotachophoresis is described in papers I and III. According to the LKB instruction book the fraction sampling procedure shall be started 1 kV before the sample reaches the detector. With the present construction at the terminating side, the section between the membrane and the valve can only be cleaned and filled with fresh electrolyte, if the membrane is unscrewed between every run. If that is not done, varying end-point voltage is obtained due to air bubbles and/or biological material. A thermal detector is now mounted "upstream" from the UV-detector on the Tacofrac plate and in this way the interval between the start and the fraction sampling procedure can be kept constant. The consumption of the cellulose acetate strip is thereby decreased.

Quantitation with a UV detector is done by measuring zone length or peak area. With the chromatography integrator, calibration and quantitation were made easier. To adapt the integrator for the UV signal, the output voltage had to be changed. The integrator is programmed to hold baseline and separate peaks by perpendicular cleavage. Calibration curves are made from 25-200% of the concentrations normally occurring in liver extracts. The correlation coefficients are better than 0.9995 for the 14 compounds examined so far. In the work with the other cell systems, new calibration curves were necessary for some compounds because their concentrations were below the calibrated interval. These curves differed somewhat from those obtained for liver extracts. As pointed out by Wielers (11) and Woledge (12), the UV-detector of isotachophoretic equipment often measures the transmitted light intensity and the recorder indicates an absorption signal equal to 100% - transmittance. With a transmittance/absorbance converter the area measured with the integrator will be linear in the whole range from small and pointed to broad and rectangular peaks. There is thus a need for a lin-log converter.

In 1965 Vestermark (13) suggested the addition of intermediate-mobility compounds which he named spacers. The spacer solution can either be a continuous mobility gradient, or composed of several discrete spacers. The optimal selection of discrete spacers is a timeconsuming trial and error work. Nevertheless the discrete spacers are to be preferred to solutions with continuous mobility gradients to improve the UV detectability. Table 2 in paper I, describing suitable spacers, has been extended and the present results are shown in a table 1.

There are two main extraction methods for nucleotides: 1) with perchloric acid, and 2) with methanol and EDTA. The latter method, often used for muscle tissue, has the disadvantage that the nucleotide stabilizing EDTA gives a broad and interfering zone between GTP and UDP-glucuronic acid in the isotachogram. There is also a broad complex-zone of EDTA which disturbs the separation of compounds like FAD, NADH, CDP and NADP. With perchloric acid extraction these two disadvantages are avoided, but even after precipitation with KOH and centrifugation at -20°C , perchlorate ions remain in solution. In the electrolyte system used, this ion moves in front of the nucleotides and does not interfere. The perchlorate ions increase, however, the molarity of the solution and

Table 1. Extended list of suitable spacers (from table 2, paper I)

UV-absorbing compounds	Spacers behind
1. UTP	hydrochloric acid, perchloric acid, oxalic acid (5%) ^a , sulfurous acid, pyrophosphoric acid
2. GTP	oxalacetic acid (10%), phosphoenolpyruvic acid ^b
3. maleic acid	pyruvic acid (3%), formic acid, tartronic acid,
4. UDP-glucuronic acid	phosphoglyceric acid, EDTA
5. fumaric acid	tartaric acid ^b
6. OMP	malonic acid, chloroacetic acid ^b , glucuronic acid-1-phosphate, dichloroacetic acid
7. ATP	fructose-1,6-diphosphoric acid
8. UDP	trichloroacetic acid ^b , EGTA
9. CTP	α -ketoglutaric acid, glyoxylic acid, phosphoric acid, 6-phosphogluconic acid
10. GDP	
11. UDP-xylose	
orotic acid	glucaric acid ^b
12. ADPRP, NADPH	
13. UDP-hexose	citric acid, malic acid, dihydroorotic acid (2%)
14. Acetyl CoA	
15. UDP-N-acetylhexos-amine	glycolic acid ^b
16. Unknown	acetoacetic acid, phosphocreatine, dihydroxyacetone phosphoric acid, glyceric acid ^b , β -glycerophosphoric acid
17. ADP	mandelic acid (5%), lactic acid, α -glycerophosphoric acid
18. FAD	N-acetylglutamic acid
19. ADPR, NADH	glucuronic acid ^b
20. CDP	α -OH-butyric acid
21. NADP	aspartic acid, glucose-6-phosphoric acid, fructose-6-phosphoric acid, α -OH-valeric acid, α -OH-isovaleric acid ^b
22. UMP	gluconic acid ^b , N-acetyl-glucosamine-1-phosphoric acid ^b
23. XMP+unknown	succinic acid, α -OH-caproic acid
24. IMP	sialic acid ^b
25. GMP	
26. cAMP, FMN	glutathione (red. form), glutaric acid, phenylacetic acid (3%)
27. UDP-galactosamine	
28. ascorbic acid	glutamic acid, adipic acid
29. AMP	acetic acid ^b , pimelic acid, laevulinic acid ^b
30. NAD	propionic acid, butyric acid
31. nicotinic acid	
32. CMP	caproic acid

Note. The right column gives compounds tested up to now. This table is only valid for the electrolyte system used in this work at pH 3.89

^aThe figures within parentheses give uv-abs. at 254 nm.

^bSpacers of special interest because they increase UV detectability where naturally occurring spacers in the extract are lacking or are in too low concentration.

thus tend to overload the system at a lower injection volume. In 1979 Verheggen et al (14) presented a column-coupling system with several advantages. In our isotachophoretic system the perchlorate zone could be trapped in the preseparation chamber and, as a consequence, the sample load could be increased. The model was adapted to the LKB Tachophor as described in paper IV. This system is still under modification and instead of the narrow bifurcation channel, as suggested by Verheggen et al (14), a valve is now tested. The problem of contamination when the sample is injected between the leading and terminating zone, is also under investigation.

The equipment of isotachophoresis is mainly used for separation of biological material and precautions concerning the maintenance must be taken. For instance the teflon capillary must be rinsed periodically with 25 ml 30% ethanol. When the capillary plate is mounted, the whole system is filled with 0.5 M urea, which is amply washed with water after 10 min. The procedure is repeated in the same way with 0.5 M NaOH and with 0.5 M HCl. The membrane of the leading side is changed at the same time.

In conclusion isotachophoresis has many advantages; technical simplicity, rapidity, low usage cost and easy quantitation. One drawback is the high cost of the equipment. As a new method in the analytical arsenal there will hopefully be further improvements such as dual wavelength detection combined with conductivity detection, better electrolyte systems, thinner capillary tubes, increased automation and computerization. Many of these improvements, proposed by Everaerts group in Holland (14-16), exist but are not commercially available.

BIOLOGICAL RESULTS

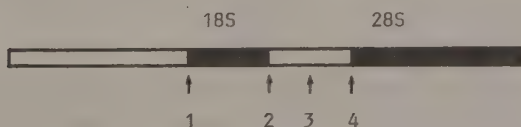
Sampling (papers I, II).

The measurement of physiological concentrations of labile metabolites is a problem which has been investigated by many authors. The original freeze-clamping method described by Wollenberger et al (17) makes it necessary to expose the organ. This can be achieved by anaesthesia, neck fraction, decapitation, stunning or the double-hatchet method (18). In paper I the double-hatchet method was tried but insufficient reproducibility was achieved in the levels of the nucleotides. Inhalating anaesthetic agents such as diethylether and divinylether were investigated (paper II), and divinylether was shown to be an excellent alternative for short operations. In subsequent experiments, with e.g injection in arteria hepatica, extended anaesthesia is necessary. Several anaesthetics are now under investigation e.g halothane, efrane and forane. It is possible that, despite minimal metabolism in the liver, as e.g. for forane (19), changes may occur in the liver due to anoxia. For example, the liver lysosomes are very sensitive to anoxia leading to release of several hydrolytic enzymes, e.g. β -glucuronidase (20,21), into the cytoplasm. This can explain the decreased UDP-glucuronic acid pool followed by elevated values of ascorbic acid in the rat liver (own observations) and in the urine following anaesthesia (22). A possible way to prevent anoxia is to ventilate artificially to maintain pO_2 and pCO_2 .

Effects of fluorosubstituted pyrimidines (paper III)

Introduction: Miller et al (23) reported in 1951 that the effect of hepatocarcinogenic aminoazodyes could be increased with substitution of a fluoro atom. In 1954 Rutman et al (24) showed that uracil was utilized more efficiently for RNA and DNA synthesis in rat hepatoma than in normal liver. These two works among others inspired Heidelberger and colleagues to synthesize 5-fluorouracil. They published their pioneering results (25) in 1957. Eidinoff et al (26) reported decreased incorporation of uracil and orotic acid into RNA after fluoropyrimidine treatment. Heidelberger et al (27) confirmed this finding in 1958 and demonstrated that fluoropyrimidines inhibited the methylation of deoxyuridylic acid to thymidylic acid. Independently, Aronson (28), Gros et al (29) and Kemper (30) demonstrated that fluoroanalogues formed abnormal ribosomes in *E. coli*. In 1966 Stenram (31) described enlarged nucleoli in the liver cells of rats after fluorouracil treatment and later, in collaboration with Willén (32,33), a delayed processing of nucleolar RNA and prevention of the appearance of cytoplasmic RNA and ribosomes. Wilkinson et al (34) and Hadjiolov et al (35) extended, in rat and mice, respectively, the understanding of the function of fluoropyrimidines in RNA processing. It was proposed that the inhibition of the maturation process from 45S RNA be due to changes in the RNA endonuclease attack, resulting in increased attack at site 2, possible attack at site 3 and reduced attack at sites 1 and 4 (36) (fig.2).

Fig. 2. Sites of endonuclease attack on a 45S RNA



The transcription of 45S RNA is regulated either at the initiation step, suggested by e.g. Mahon et al (37) or at the propagation/elongation step, suggested by e.g. Dauphinais (38). The effect of fluoropyrimidine treatment on transcription is not well known. Hadjiolov et al (36,39) found an unchanged transcription rate, while Wilkinson et al (40) found a time dependent inhibition.

Results and discussion: Male white rats were given either orotic acid or 5-fluoroorotic acid 60 min, and ^3H -orotic acid 3 1/2 min before sacrifice. The RNA synthesis measured as nmol of UTP entering RNA-UMP/g wet liver tissue was significantly lower in the treated rats compared to controls. A possible explanation for this is that treatment with fluoropyrimidines creates a block in the processing followed by feedback inhibition of the transcription. This idea was recently supported by Iapalucci-Espinoza and Franze-Fernandez (41), who found that the control of rRNA processing and transcription was coordinated in cells with high rates of rRNA synthesis. As mentioned in paper III our results are dependent on the specific activity of (F)UTP in the nucleus being equal to the specific activity of (F)UTP in the whole cell. Siebert (42) and Stärk *et al* (43) found no differences in nucleotide pools in the nucleus compared to the pools in cytoplasm. On the other hand many authors propose a compartmentation of pyrimidine triphosphates (for review, see Keppler and Holstege (44)). Wiegers *et al* (45) and Genchev *et al* (46) even suggest compartmentation within the nucleus. It is our intention to study these problems.

The flow of labelling from UTP to UDP-hexose, UDP-N-acetylhexosamine and UDP-glucuronic acid was also investigated. The findings revealed no significant differences between animals treated with fluoroorotic acid or orotic acid. The (F)UTP-pool increased to the same extent compared to animals given neither orotic acid nor fluoroorotic acid. The specific activity of (F)UTP was similar and the labelling of (F)UDP-hexose and (F)UDP-N-acetylhexosamine was equal. The interest in UDP-sugars has increased because the fluoropyrimidines incorporated in (F)UDP-hexose and (F)UDP-N-acetylhexosamine can be reutilized and in this way prolong the effect of treatment with fluoropyrimidines (47).

A major finding in the experiments with the sub-nuclear fractions was an increased RNA/DNA ratio in the nucleolar fraction. The specific nucleolar RNA labelling was decreased but per μg DNA it was essentially unaltered. The specific labelling of RNA/ μg DNA was very similar in the nucleolar and the nucleolar-free fractions suggesting an equal transcription rate. Our suggestion that false RNA may loosen prematurely from DNA and accumulate in the supernatant is derived from the decreases of specific RNA activity/ μg DNA in the nucleolar and nucleolar-free fractions

together with increase of specific RNA activity in the supernatant fraction with no change in RNA/DNA ratio. In addition, Rungger and Crippa (48) observed accumulation of free ribonucleoprotein fibrils and increased amount of low-molecular spacer transcripts when the Miller method (49) was used on cells treated with fluorouridine.

Concluding remarks: Today the fluoro analogues are of increasing interest as chemotherapeutics in combination with other substances e.g. N-(phosphonacetyl)-L-aspartate (PALA) and methotrexate or with such cancertherapeutic methods as intraarterial injection of microspheres and ligation of arteries. Our laboratory is now involved in two experiments in this area, a) the combined effects of PALA and glucosamine to increase the uptake of fluorouridine in adenocarcinoma, b) the effect of microspheres on the uptake and distribution of fluoropyrimidines in the liver.

Despite extensive investigation over a period of more than 25 years, it is still unclear whether thymidylate synthetase inhibition or inhibition of RNA maturation is the main effect of the fluoro compounds. In some cases, an excellent correlation exists between the antitumor properties and the formation of FdUMP (50-53), and in other cases there is a correlation with the incorporation into RNA (52,54,55).

Sawyer et al (56) have found that DNA synthesis measured as ^{32}P incorporation was not shut off until both the de novo and the salvage pathways were inhibited. Thymidylate synthetase was inhibited immediately because of interaction with FdUMP. The complete inhibition of DNA synthesis also required thymidine kinase inactivation. This occurred later and was correlated to the inhibition of ribosomal RNA synthesis and thereby inhibition of enzyme production. Sawyer et al also point out that DNA synthesis could be disturbed by the FUMP content in a pre-formed RNA primer, which is thought to function as a template for DNA synthesis (for review see 57).

DNA-repair after γ -radiation and hyperthermia (paper V)

Introduction: Studies on DNA repair mechanisms have intensified in recent years. One reason for this is the recognition that the development of cancer following induction of DNA damage is associated with inability of cells to repair DNA lesions. The latter function fails in some inherited human disorders e.g xeroderma pigmentosum, ataxia telangiectasia and Fanconi's anemia, where the affected individuals are cancer prone (58). It is also possible to take advantage experimentally of the DNA repair mechanism when developing cancer treatment protocols. For example radiation in combination with hyperthermia and cytotoxic agents might be utilized to further suppress the DNA repair synthesis. Estimation of the risk in humans to develop cancer may also be possible using screening methods based upon measurement of carcinogen-induced unscheduled DNA synthesis (59).

Many environmental factors, such as UV-light, γ -radiation and chemical substances can damage DNA. Examples of DNA damages are thymidine dimer production, crosslinks, single or double strandbreaks, base loss and alkylation. During evolution the living organism has developed DNA repair processes to keep the genetic code. Different mechanisms for removal of different DNA damages have been proposed (for review see (60)):

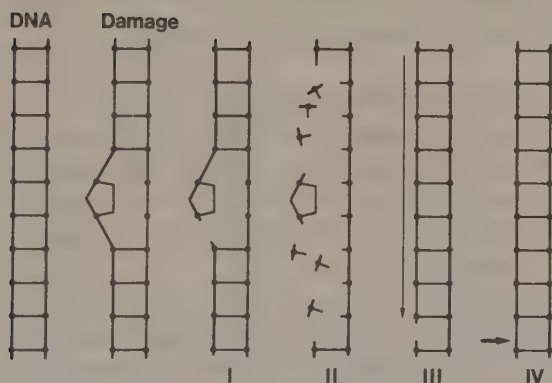
1. Photo reactivation
2. Excision repair (see fig. 2)
3. Post-replication repair
4. Repair of strandbreaks

In 1956 Roitt (61) showed that NAD^+ was decreased after addition of cytotoxic agents, and many workers have observed the NAD^+ depletion after ionizing radiation (62,63). It was not until 1975 that NAD^+ depletion, ADP-ribosyl transferase, which catabolizes NAD^+ to protein-bound poly(ADP-ribose) and this phosphorylated protein were linked to the DNA repair process. (64-66).

Ionizing radiation in cancer therapy is well known, but in recent years the combined therapy with hyperthermia (temperatures $> 42^\circ\text{C}$) and γ -radiation has been shown to increase the tumoricidal effect. The under-

Fig. 2. The excision repair model:

- I: A DNA endonuclease recognizes the damage and makes an incision.
- II. A DNA exonuclease removes a sequence around the damage.
- III: A DNA polymerase synthesizes the missing sequence using the single stranded DNA as template.
- IV: A DNA ligase completes the repair.



lying mechanism is unclear and many effects have been proposed: decreased pO_2 and cell pH, altered protein and nucleic acid synthesis, changes in cell membrane and lysosomes, and effects on cell cycle phases and growth rate. Many of these effects are directly or indirectly linked to oxidative phosphorylation and changes in chromatin structures. The enzyme, ADP-ribosyl transferase, is known to alter the chromatin structure and the substrate for this enzyme, NAD^+ , is dependent on ATP for its synthesis. Consequently, it seemed logical to study ATP, energy charge, NAD^+ recovery and unscheduled DNA synthesis after γ -radiation and/or hyperthermia.

Results and discussion: Human mononuclear leukocytes in vitro were employed. The unscheduled DNA synthesis (UDS) was measured after heat treatment at different temperatures (37°C - 45°C) for 45 min followed by exposure to a radiation of 30 Gy. The UDS level decreased at higher temperatures and at 42.5°C it was 30% lower than at 37°C .

After 45 min at 37°C followed by γ -radiation, the NAD^{+} pool in the leukocytes decreased to 20% of that in controls (for definition of controls, see Methods) but it recovered completely within 5 h. The same NAD^{+} depletion/ NAD^{+} recovery has been previously described in mouse leukaemic cells, L 1210 (67). Combined hyperthermia and γ -radiation decreased the NAD^{+} recovery, e.g. at 42.5°C the NAD^{+} level only recovered to 70% of controls even after 8 h incubation.

These findings combined with the viability experimental data, (fig.7, paper V), revealing no difference in cell death at 37°C and 42.5°C within 20 h after heat treatment alone, indicate that the DNA polymerase β activity (estimated by UDS) as well as the ADP-ribosyl transferase activity (estimated by NAD^{+} depletion) become critical for DNA repair after combined treatment.

ATP levels and ATP/ADP ratios after 8 h incubation are shown in table 2 (values from figs. 4 and 5, paper V). The sharp decrease of ATP at 44°C (to 25% of controls) in combination with a delayed increase of cell death at 44°C (10% dead cells at 10 h and 40% at 20 h, fig. 7, paper V) indicates an effect of hyperthermia on cell survival. This is in accordance with results of Laval and Michel (68), showing tumor regression after heat treatment. The ATP/ADP ratios were not reduced to the same extent which probably reflects an attempt to keep the energy charge in spite of ATP loss.

After combined hyperthermia/radiation treatment there was an initial reduction of the ATP levels. After irradiation at 37°C and 42.5°C the ATP pools recovered and after 3 h and 6-8 h, respectively, they were less than 10% below the ATP levels in non-irradiated cells. After irradiation at 44°C the ATP pool never recovered. The ATP/ADP ratios showed a pattern similar to the ATP levels except at 44°C , where recovery at a low level could be observed.

Table 2. ATP and ATP/ADP ratios at 8 h incubation (values from figs. 4,5 paper V)

	ATP		ATP/ADP	
	% of controls		% of controls	
	0 Gy	+30 Gy	0 Gy	+30 Gy
37°C	72	65	80	67
42.5°C	65	55	80	67
44°C	25	12	50	38

Table 3. Viable cells after 50 h incubation (values as % of controls, from fig. 7, paper V)

	37°C	42.5°C	44°C
0 Gy	75	65	43
+30 Gy	45	31	20

The data in table 2 show that irradiation at 37°C affected the ATP pool less than heat treatment at 44°C and to the same extent as heat treatment at 42.5°C. Table 3 shows a similar cell killing effect within 50 h after irradiation at 37°C and after heat treatment at 44°C. In conclusion, hyperthermia, but not γ -radiation, decreases the ATP pool sharply while DNA polymerase β activity (estimated as UDS) and ADP-ribosyl transferase activity (estimated as NAD^+ pool) are unaffected by hyperthermia treatment. These findings suggest two mechanisms of cell killing for heat treatment and γ -radiation: γ -radiation may mainly interfere with DNA metabolism, while hyperthermia may affect the cell less specifically with the oxidative phosphorylation as the main target.

After combined hyperthermia/radiation treatment there is a synergistic effect where the two suggested mechanisms are linked by ATP. A sharp reduction of ATP limits NAD^+ regeneration, leading to reduced amount of ADP-ribosylation and inhibition of DNA repair. Kaufmann *et al* (69) suggest that ATP and other nucleoside triphosphates also actively pre-

vent degradation of newly synthesized DNA and stimulate DNA repair. The importance of ATP depletion in inhibiting DNA repair is under further investigation. In a report to be published we found that added adenosine is phosphorylated to ATP after 5 h of incubation at 37°C, 42.5°C and at 37°C + 30 Gy while 42.5°C + 30 Gy decreases the ATP pool compared to controls. These data suggest a profound interference in the phosphorylation mechanism in cells treated with hyperthermia in combination with γ -radiation, enhancing cell killing.

Concluding remarks: The understanding of the control and kinetics of DNA repair processes is important, and research in this area is active. As may be expected in fields where much is being done, there are conflicting points of view. For example, in different reports from 1980 to 1982 the enzyme converting NAD^+ to nicotinamide and protein-bound poly(ADP-ribose) has been given several names: ADP-ribosyl transferase, poly(ADP-ribose) synthetase, ADP-ribose polymerase, poly(ADP-ribose) polymerase and mono(ADP-ribose) transferase, and they are probably the same enzyme. Other controversies regard the effects of nicotinamide and benzamide which are inhibitors of ADP-ribosyl transferase. In rat liver cells and resting lymphocytes these substances increase the UDS (70,71) but in L 1210 cells they inhibit excision repair and survival (72).

Studies, which to some extent are based on inhibitors of ADP-ribosyl transferase, have given rise to two main theories. a) Miwa et al (73) suggest that inactivation of ADP-ribosyl transferase and thereby decreased phosphorylation stimulates DNA endonuclease to make more cuts for repair synthesis to start. b) Creissen and Shall (74) find a correlation between active ADP-ribosyl transferase and efficient DNA excision repair by activation of DNA ligase.

ADP-ribosyl transferase is involved in cell differentiation (75) and gene expression (70) as well as in the repair of chemically or physically damaged DNA.

The turnover of glycosaminoglycan precursors and influence thereon of glucocorticoids.

The fibroblast is the most common cell in fibrous connective tissue and actively secretes collagen, proteoglycans and hyaluronic acid into the matrix. Proteoglycans are composed of protein and covalently bound polyanions as dermatan sulfate or heparan sulfate. The repeating disaccharide units are shown in table 4. The degree of sulfation in the sulfated glycosaminoglycans can vary and in dermatan sulfate there is approximately one sulfate/disaccharide while heparan sulfate has 0.5-1 sulfate/disaccharide.

Table 4. The saccharide units in glycosaminoglycans in fibroblasts.

a=dominating component, b=minor component

Glycosamino- glycans	Uronic acid	Hexosamine
Hyaluronic acid	glucuronic acid	N-acetylglucosamine
Dermatan sulfate	iduronic acid ^a glucuronic acid ^b	N-acetylgalactosamine
Heparan sulfate	glucuronic acid	glucosamine-N-sulfate ^a N-acetylglucosamine ^b

The major precursors of the saccharide units are UDP-glucuronic acid, UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine, and PAPS, which donates sulfate to e.g the sulfated glycosaminoglycans. Iduronic acid and glucosamine-N-sulfate (table 4) are formed on the polymer level by epimerization of the glucuronosyl residue and by deacetylation and sulfation of the N-acetylglucosamine residue, respectively.

In labelling experiments of glycosaminoglycans synthesis ³⁵S-sulfate and ³H-glucosamine are most commonly employed. They are mainly incorporated into PAPS and UDP-N-acetylhexosamine, respectively.

With the increased use of glucocorticoids as anti-inflammatory drugs for topical application in skin diseases, side-effects, as thinning of skin and impaired wound healing were observed. At the cell and molecular level, a spectrum of effects has been found: inhibition of cell proliferation and mitosis (76), effects on prostaglandins and leukotrienes (77), decrease in glycosaminoglycan synthesis (78), changes in RNA synthesis (79), inhibition of collagen production (80), and induction of some other proteins (81). A main approach in studies of the mechanisms behind the synthesis of glycosaminoglycans and drug action is in vitro systems. Fibroblasts in culture are useful for this purpose.

In a recent study on fibroblasts in culture (82), my collaborators found a discrepancy in the incorporation of ^{35}S -sulfate and ^3H -glucosamine into the sulfated glycosaminoglycans after glucocorticoid treatment. It was proposed that this effect was due to an oversulfation or a change in the precursor pools. This led to the present investigation of the nucleotide pools and the labelling of precursors in cultured fibroblasts.

Results and discussion: An extraction method for nucleotides and nucleotide sugars was adapted for fibroblasts in culture. Quantitation by isotachopheresis revealed a high energy charge (0.9) and minimal breakdown of UTP and UDP-sugars, indicated by a low UMP pool. Important precursors of glycosaminoglycans such as UDP-glucuronic acid and UDP-N-acetylhexosamine were easily quantitated, while the PAPS pool was too small to quantitate and UDP-hexose interfered with NADPH. The problem with a mixed zone of NADPH and UDP-hexose has recently been solved by lowering the pH to 3.87 in the leading electrolyte and by using saccharic acid as spacer. Two discrete peaks are obtained.

The labelling experiments with ^{35}S -sulfate showed an equilibration with PAPS attained in less than 10 min. The ratio of ^{35}S -sulfate to ^{35}S -PAPS was constant (92/8) from 5 min to 8 h. These results reveal that ^{35}S -sulfate is a useful precursor in studies of the synthesis of sulfated glycosaminoglycans. The equilibration time for UDP-N-acetylhexosamine using ^3H -glucosamine as precursor was about 4 h. The same results were obtained with ^{14}C -glucose. As there was no build-up of intermediates, this indicates that the large pool size and slow turnover

of UDP-N-acetylhexosamine are the main reasons for the long equilibration time.

More than 95% of the acid soluble activity using ^3H -glucosamine was incorporated into UDP-N-acetylhexosamine. In the isotachophoretic analysis the remainder was found in two areas corresponding to N-acetylhexosamine-phosphate and CMP-sialic acid.

Table 5. Nucleotide pools in cultured fibroblasts after 48 h preincubation with or without 10^{-5} M budesonide (mean $\pm$ s.e.m)

	Control n = 4	Budesonide n = 4	
UTP	186 $\pm$ 7.4	148 $\pm$ 5.4	**
GTP	219 $\pm$ 4.3	207 $\pm$ 8.8	
UDP-glucuronic acid	38 $\pm$ 1.3	50 $\pm$ 2.1	**
ATP	948 $\pm$ 14.6	905 $\pm$ 40.5	
UDP-N-acetyl- hexosamine	589 $\pm$ 40.5	833 $\pm$ 31.9	**
ADP	113 $\pm$ 3.9	119 $\pm$ 12.4	
AMP	13 $\pm$ 0.8	23 $\pm$ 3.6	
NAD	206 $\pm$ 5.6	214 $\pm$ 4.5	

** = $0.001 < p < 0.01$

Preliminary data from our group (Särnstrand *et al*, to be published) showed significant differences in the nucleotide pattern in fibroblasts treated with 10^{-5} M budesonide (83) (Draco AB, Sweden) for 48 h compared to controls. A decrease of UTP (20%) and an increase of UDP-glucuronic acid (32%) and UDP-N-acetylhexosamine (41%) were observed while other quantified nucleotides showed no significant changes. The increase of the two precursors is probably due to an inhibition of glycosaminoglycan synthesis by glucocorticoids. One explanation for the decrease of UTP may be a slower utilization of glycosaminoglycan precursors, giving a decreased reutilization of UDP units to UTP. Further investigation of the turnover of the precursor pools, in the presence and absence of

budesonide, may reveal the reason for the changes.

Concluding remarks: In aging and in certain pathological conditions such as atherosclerosis, rheumatic disorders and pulmonary fibrosis there are changes in the connective tissue. One approach to the understanding of the regulatory mechanisms behind such changes is in vitro studies of fibroblasts. In such systems the effects of e.g. glucocorticoids and prostaglandins on the glycosaminoglycan synthesis can be more easily controlled.

The effects of glucocorticoids are complex. One of the main reasons is the translocation to the nucleus of an activated receptor-mediated form. There is increasing evidence that localization and binding to chromatin DNA initiates new mRNA synthesis (79) and suppresses some other ongoing mRNA synthesis with secondarily alterations in protein synthesis. In the fibroblasts this results in a decrease in collagen and glycosaminoglycan synthesis.

The effects of prostaglandins on glycosaminoglycan synthesis are stimulatory, $\text{PGF}_{2\alpha}$ being the most potent agent. Muroto et al (84) have shown that the production of hyaluronic acid is stimulated most and that $\text{PGF}_{2\alpha}$ induces hyaluronic acid synthetase activity by activation at the DNA template level.

Some of the effects of glucocorticoids and of prostaglandins are opposing and, in addition, glucocorticoids interfere with prostaglandins by inhibiting arachidonic acid production. Further studies of such events using hormones and drugs may increase the understanding of the regulation of the turnover of compounds in connective tissue. In combination with experiments using different cell types, cells from different organs or species and from donors of differing ages, therapeutic agents may be found.

SUMMARY

This work was undertaken to investigate the nucleotide metabolism in rat liver, human mononuclear leukocytes and fibroblasts with isotachopheresis.

An in situ sampling method for rat liver was worked out. Divinylether was shown to be a suitable anaesthetic agent for short operations. An extraction method for acid-soluble metabolites was adapted to the lability of the nucleotides and for isotachopheresis. The preparation was done at -20°C .

An electrolyte system for separation of liver nucleotides with isotachopheresis was developed. Different non-UV-absorbing substances were investigated as spacers to increase UV-separation. A column-coupling system was connected to the tachophor capillary to trap fast moving ions and to increase sample load.

The effect of fluoroorotic acid on the early labelling of nucleotides and RNA in the liver of rat was examined by giving this substance or orotic acid 60 min, and ^3H -orotic acid 3 1/2 min before sacrifice. The RNA synthesis (measured as nmol UTP entering RNA) was decreased after fluoroorotic acid treatment. This indicates a decreased transcription rate of RNA. The nucleolar fraction and the nucleolar-free part of the nucleus were affected to the same extent. Treatment with fluoroorotic acid also increased the RNA/DNA ratio of the nucleolar fraction, an effect of a delayed processing of ribosomal RNA.

In experiments with human mononuclear leukocytes in vitro, the DNA repair synthesis was investigated after exposure to hyperthermia and/or γ -radiation. Combined treatment resulted in decreased DNA polymerase activity (estimated as unscheduled DNA synthesis) and decreased ADP-ribosyl transferase activity (estimated as NAD^+ depletion) compared to γ -radiation only. The oxidative phosphorylation seems to have a central role in DNA repair. In an experiment adenosine was added to the cells after treatment. The ATP pool increased due to phosphorylation of adenosine after treatment with either hyperthermia or radiation but decreased after combined treatment.

Embryonic human fibroblasts in culture were used in labelling experiments to study the equilibration of precursor pools of glycosaminoglycans. The PAPS pool was equilibrated within 10 min after addition of ^{35}S -sulfate, while the UDP-N-acetylhexosamine pool required 3-5 h to attain steady state after incubation with ^{14}C -glucose and ^3H -glucosamine. In conclusion, labelling experiments with precursors having different equilibration times ought to be interpreted with care. In experiments with budesonide, a potent glucocorticoid, the pools of UDP-glucuronic acid and UDP-N-acetylhexosamine increased compared to controls. This probably reflects the inhibited glycosaminoglycan synthesis.

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A Method for Measuring Nucleotides in the Liver of Rat with Isotachopheresis

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A cold perchloric acid-methanol extraction of the liver of rat, followed by KOH neutralization, was adapted for direct use in analytical isotachopheresis with uv detection. Separation of nucleotides with isotachopheresis is convenient, because sample injection of a few microliters results in good reproducibility and resolution down to a 100-pmol level within 45 min. If two uv-absorbing compounds are not readily separated, the addition of a non-uv-absorbing compound, a spacer, with a mobility inbetween, can improve uv detection. A measurement of a pooled extract of three rats with isotachopheresis gave the following concentrations of ATP, ADP, AMP, and UTP: 3000, 715, 160, and 360 nmol/g wet liver tissue, respectively. Such information was reached in 45 min and the ability to determine radioactive labeling will be potentially useful in the study of, e.g., energy charge and RNA synthesis with orotic acid as radioactive precursor.

For the past several years our laboratory has studied the effect of protein deficiency and cytostatics on the RNA metabolism of the liver. The importance of studying the RNA precursors after radioactive labeling led to the investigation of capillary isotachopheresis for determination of nucleotides. In 1975 Dunn *et al.* (1) used isotachopheresis for separating acid-extracted nucleotides from liver, but the technique at that time was not satisfactory for the present purpose. Separation of nucleotides in muscle extract (2-4) has shown good reproducibility and resolution. The aim of the present study is to adapt isotachopheresis to the more composite liver extract. When analyzing biological material, a number of problems arise, e.g., tissue sampling, extraction, and large differences in the concentrations of substances. The method described considers such problems and all volumes are adapted for separation with isotachopheresis.

MATERIALS AND METHODS

Chemicals. All nucleotides and their derivatives for determining the peaks in the uv

absorbing scan, β -alanine, and *n*-caproic acid were purchased from Sigma Chemical Company, St. Louis, Missouri. Other materials were purchased as follows: triethanolamin, Merck, Federal Republic of Germany; hydroxypropylmethylcellulose (HPMC),¹ Dow Chemical Company, Midland, Michigan. All chemicals were pro analysi and used without further purification, except HPMC which was purified according to Woledge (4). The water used was treated in a Milli-Q System, giving a resistivity of 18 Mohm-cm (Millipore Corp., Bedford, Mass.).

Animals and liver sampling. Male Wistar rats weighing about 150 g were used. Unanesthetized and unstressed rats were killed at about 8 AM according to the double-hatchet method (9). Liver samples were excised and frozen between two metal blocks(5) precooled in liquid N₂, and transferred to a thermos filled with liquid N₂ for

¹ Abbreviations used: hplc, high-pressure liquid chromatography; HPMC, Hydroxypropylmethylcellulose; XMP, xanthine monophosphate; UDP-GlcUA, UDP-glucuronic acid.

TABLE 1
OPERATIONAL SYSTEM AT pH 3.89 FOR SEPARATION
OF NUCLEOTIDES

Solvent: H ₂ O		
Electric current at registration: 37.5 μ A		
	Capillary	Anode compartment
Leading electrolyte		
Anion	Cl ⁻	Cl ⁻
Concentration (M)	0.005	0.005
Counterion	β -Alanine	β -Alanine
Additive	HPMC	—
pH at 20°C	3.89	3.89
Terminating constituent	Caproic acid	
Concentration (M)	0.005	
pH correction	None	

preservation until the deproteinization. The time from hatchet stroke to freezing was 3–4.5 s.

Extraction. Deproteinization was done in a room thermostated at +4°C. The frozen liver was transformed to a porcelain mortar and pulverized, while liquid N₂ was continually added. The powder was quickly transferred to a centrifuge tube, with 2.5 ml of 0.7 M perchloric acid and 20% (v/v) methanol, standing on a balance. Perchloric acid with methanol was added to obtain 7 ml/g liver. The sample was immediately homogenized for 30 s with a high-speed blade homogenizer (Measuring and Scientific Equipment Ltd., England) in a salt-ice bath at -8°C. The tube was centrifuged for 1 min at 39,000g and -10°C (setting temperature) in a Sorvall RC2-B centrifuge with an SS-34 rotor (Sorvall, Newton, Conn.) Five milliliters of the supernatant was transferred to a tube with 250 μ l of 0.4 M triethanolamin buffer at pH 7.2 and 400 μ l of 7 M KOH. The sample was adjusted with 2 M KOH to pH 7.1–7.3. The neutralization was performed rapidly under magnetic stirring in a refrigerated bath circulator at -10°C (Haake, F. R. G.). The total time for the biological material in acid was less than 10 min. The sample was centrifuged

for 5 min at 8000g and -10°C. The supernatant was equally divided into small tubes and stored frozen (-80°C) for later use.

Isotachophoresis. Isotachophoresis was performed on an LKB 2127 Tachophor (LKB, Sweden) equipped with a uv lamp at 254 or 280 nm and a vacuum phototube as detector. Analytical separation was run in a Teflon capillary tube (length 43, 62, or 81 cm, i.d.; 0.5 mm), thermostated at 20°C.

The electrolyte system is described in Table 1. HPMC was used as stabilizing medium at final concentrations of 0.25, 0.50, or 0.75%. A PHM 64 research pH meter was used with a combined electrode, type GK 2321C, calibrated against standard buffers at pH's of 3.00 and 7.00 at 20°C (Radiometer A/S, Denmark).

Before starting, the Tachophor was carefully rinsed and prerun with water at 0.05 μ A until 20 kV was reached. When the system had been filled and stabilized, a volume of about 10 μ l was injected 2 mm inside the leading electrolyte and the micro-syringe was slowly withdrawn. The separations were started at 125 μ A and manually reduced to 75 and 37.5 μ A, so that the effect never exceeded 2.5 W. This precaution was taken to avoid formation of air bubbles in the capillary. During detection at 37.5 μ A, the voltage rose from 19 to 21.5 kV for the 81-cm capillary.

The chart speed was 5 cm/min. The peaks were monitored with an LDC 304:50 computing integrator (Laboratory Data Co., England), programmed to hold baseline and separate peaks by perpendicular cleavage.

Preparative isotachophoresis for radioactive determination was run in a Tachofrac kit (LKB, Sweden) described by Arlinger (6). The cellulose acetate strip (Sartorius, F. R. G.) was cut into 1-mm lengths in a stamp made in our laboratory. Each piece was put into a vial and 10 ml of Instagel (Packard, Downers Grove, Ill.) was added. The scintillation countings were made in an SL 30 liquid scintillation spectrometer (Intertechnique, France).

High-pressure liquid chromatography. Analyses of nucleotides were performed using an hplc system (Waters Assoc., Milford, Mass.). A 0.5×20 -cm column was packed with Partisil 10 SAX (Whatman, Clifton, N. J.) and maintained at 55°C . To increase separation a 1.4 mM to 0.7 M KH_2PO_4 gradient at pH 4.5 was used at a flow rate of $1.5 \text{ ml} \times \text{min}^{-1}$ according to Chapman *et al.* (7).

RESULTS AND DISCUSSION

Determination of Optimal Experimental Condition

The routine perchloric acid extraction procedure for nucleotides was improved by adding methanol (8). In addition to inhibiting enzymatic activity, this allowed the temperature to be lowered to below 0°C , further preventing metabolic changes and increasing the potassium perchlorate precipitation at neutralization. The latter favored a shorter run, a better resolution, and a larger injection volume without overloading the system.

Recovery experiments were made for controlling the stability of ATP, ADP, AMP, and UTP. The extraction was simulated for 1.0 ml 1.0 mM nucleotide with 7.0 ml of perchloric acid-methanol. ATP was shown to be the most labile with a loss of less than 1%. Control runs, with no liver extract, were also investigated (Fig. 1), and $\text{HClO}_4 + \text{KOH}$ resulted in an impurity or complex zone in front of ADP (Peak 12 in Figs. 1, 2, and 3).

Experiments were carried out at different concentrations and pH's of leading electrolyte. The conditions mentioned under Materials and Methods were finally selected in all subsequent separations.

Calibration and Quantitation

Calibration curves were made, using standard solutions of nucleotides and the areas of the uv-absorbing compounds were calculated with the integrator (see Materials

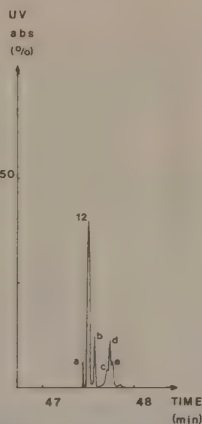


Fig. 1. Record at 254 nm of control extraction with no liver tissue for determination of impurity zones. $8 \mu\text{l}$ was injected. Peak 12 is caused by HClO_4 -KOH and the others by the electrolytes. They interfere with liver extract as follows: a, with UTP; b, with ADP; c, with XMP; d, with ascorbic acid, and e, with NAD. Tube length 81 cm.

and Methods). The tachophor scan in Fig. 4 shows good separation of a standard mixture of 12 nucleotides (1 nmol each). The zones are close to each other which distinguishes isotachophoresis from other forms of electrophoresis. Compounds such as GTP and ATP are well separated in the UV scan due to non-uv-absorbing impurities in the electrolytes or in the chemicals. To get better registration of compounds close to each other a continuous investigation is made to test non-uv-absorbing substances as spacers. Table 2 shows the present results. The mobilities of some compounds such as ATP and UDP are very similar in this system and a complete separation is at the moment difficult to obtain (Fig. 4).

Linearity and Reproducibility

Calibration curves were made by using increasing concentrations of substances of

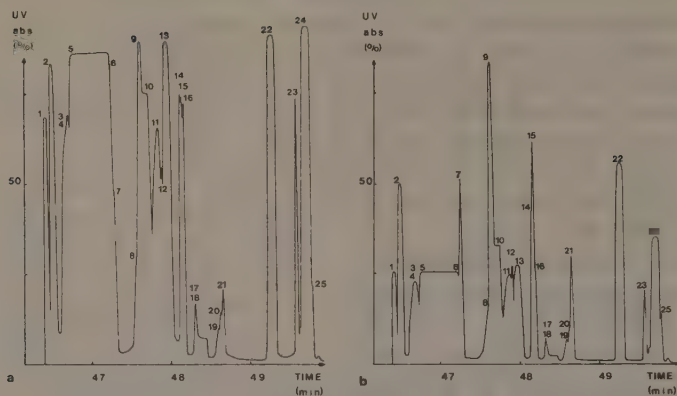


FIG. 2. Two records of the same liver extract at 254 nm (a) and 280 nm (b). 8 μ l was injected. Tube length 81 cm. 1. UTP, 2. GTP, 3. UDP-GlcUA, 4. Fumaric acid, 5. ATP, 6. UDP, 7. CTP, 8. GDP, 9. NADPH, 10. UDP-sugar, 11. UDP-N-acetylaminosugar, 12. Impurity or complex zone from HClO_4 -KOH, 13. ADP, 14. FAD, 15. NADH, CDP, 16. NADP, 17. UMP, 18. XMP + unknown, 19. IMP, 20. GMP, 21. FMN, 22. Ascorbic acid, 23. AMP, 24. NAD, 25. CMP.

analytical grade. Figure 5 shows the relation between area and amount of ATP, ADP, AMP, and UTP (for ATP the relation zone length: amount is also shown). The linearity is good with a correlation coefficient of 0.9995 or better in the range of concentrations normally occurring in the liver extracts. Ten determinations of ATP were carried out in an aqueous solution to control the reproducibility and resulted in a coefficient of variation of $\pm 2.16\%$ for 2 μ l of 0.5 nmol ATP/ μ l.

Analysis of Liver Extract

Figure 2 shows a typical scan of liver extract. The profiles presented in the figure are representative of the results obtained in more than 100 experiments. Variations are due to the liver sampling and concern most visible the ATP/ADP/AMP ratios. Nucleotide materials were identified on the basis of the following criteria: (a) A comparison of the uv-absorption at 254 and 280

nm (see Fig. 2). (b) Coincident mobility with authentic samples added to the tissue extract (see Table 3 for ATP). (c) Incorporation of radioactivity. Administration of [^{14}C]orotic acid, to be used in our future studies of RNA metabolism, 5–20 min before killing, resulted in high specific activity in UTP, UDP, UMP, UDP-glucuronic acid, UDP-sugar and UDP-aminosugar, and no activity in other peaks. (d) Comparison of results with isotachopheresis and hplc (7). With the same liver extract, identical results were obtained except for a lower ADP concentration with hplc. (e) Comparison of results with literature (9–14) showed good agreement.

Further investigations with enzymatic modifications are now being tested for substances where a quantitative interference may occur, e.g., UDP-glucuronic acid, CTP, and UDP-N-acetyl-aminosugar.

Figure 2 reveals that the uv separation is good for most of the substances because of naturally occurring spacers, e.g., phos-

phoric acid behind CTP and lactic acid behind ADP. The separation is dependent on the volume injected. A small volume ($=4 \mu\text{l}$) gives poor separation because the amount of spacers becomes too small. A volume exceeding $25 \mu\text{l}$ gives poor separation because the system is overloaded. The separation can be improved by adding spacers. CTP is for example easily separated from ATP/UDP with trichloroacetic acid (Fig. 3). The reproducibility for 10 repeated runs was good, with a coefficient of variation below 4.0% for all the peaks except ascorbic acid, which is very sensitive for exposure to room temperature, with a loss of over 70% during this experiment. As a result of this experiment, the samples are stored at -80°C and kept at -10°C during the day of use, except for the short period when the microsyringe is filled. The

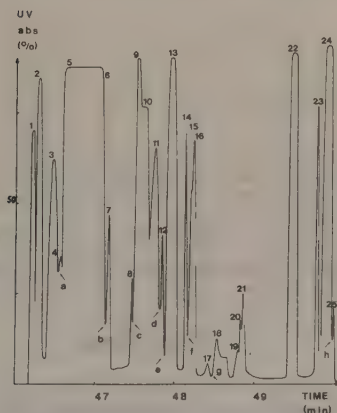


FIG. 3. Record at 254 nm of the same liver extract as in Fig. 2 but with eight spacers added at a concentration of 0.5 mM. $8 \mu\text{l}$ liver extract + $2 \mu\text{l}$ spacer solution was injected. Tube length 81 cm. a, tartaric acid; b, trichloroacetic acid; c, glucaric acid; d, glycolic acid; e, glyceric acid; f, glucuronic acid; g, gluconic acid; h, propionic acid. The results can be compared with those in Table 2. The numeration refers to Fig. 2.

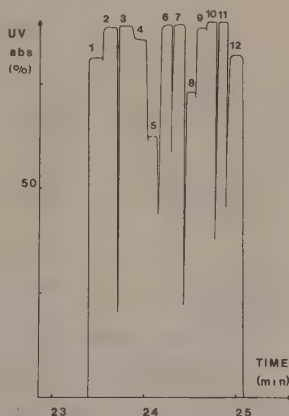


FIG. 4. Record of a mixture of 12 standard nucleotides (1 mM of each). $12 \mu\text{l}$ was injected and detected at 254 nm and $50 \mu\text{A}$. Tube length 62 cm. 1. UTP, 2. GTP, 3. ATP, 4. UDP, 5. CTP, 6. GDP, 7. ADP, 8. CDP, 9. UMP, 10. GMP, 11. AMP, 12. CMP.

samples are discarded at the end of the day. Runs with different amounts of liver extract from 0.5 to $6 \mu\text{l}$ were also made in order to find out if there was a linearity as good as with pure nucleotides. The correlation coefficient for ATP, ADP, and AMP was 0.9998, 0.9995, and 0.9973. The results showed good agreement with those in aqueous solution. A recovery experiment was made by running 1 nmol ATP pro analysis with 4, 6, and $8 \mu\text{l}$ of liver extract. The results are shown in Table 3.

Uridine compounds are of special interest to us, as we plan to study the RNA synthesis with the use of orotic acid as precursor. The adenine nucleotides are always important because it is easy to control the energy state and extraction condition by comparing ATP, ADP, and AMP. Those substances have therefore been specially considered. The concentrations of ATP, ADP, AMP, and UTP from the pooled extract of three rats were 3000, 715, 160,

TABLE 2
SURVEY OF SUITABLE SPACERS

uv-Absorbing compounds	Spacers behind
	Hydrochloric acid, Oxalic acid (5%) ^a
	Sulfurous acid
1. UTP	Oxalacetic acid (10%), phosphoenolpyruvate
2. GTP	Formic acid, tartronic acid, phosphoglyceric acid, pyruvic acid (3%)
3. UDP-glucuronic acid	<i>Tartaric acid</i> ^b
4. Fumaric acid	Malonic acid, chloroacetic acid
5. ATP	
6. UDP	<i>Trichloroacetic acid</i>
7. CTP	Phosphoric acid, 6-phosphogluconic acid
8. GDP	<i>Glucaric acid</i>
9. NADPH	
10. UDP-sugar	Citric acid, malic acid, dihydroorotic acid (2%)
11. UDP-N-acetylaminosugar	<i>Glycolic acid</i>
12. Impurity or complex from HClO ₄ -KOH	Phosphocreatine, dihydroxyacetone phosphate, <i>glyceric acid</i> , β -glycerophosphate
13. ADP	Lactic acid, mandelic acid (5%)
14. FAD	<i>Glucuronic acid</i>
15. NADH, CDP	
16. NADP	Aspartic acid, glucose-6-phosphate
17. UMP	<i>Gluconic acid</i>
18. XMP + unknown	Succinic acid
19. IMP	
20. GMP	
21. FMN	Glutathione (red. form)
22. Ascorbic acid	Glutamic acid, adipic acid
23. AMP	Acetic acid, pimelic acid, <i>laevulinic acid</i>
24. NAD	<i>Propionic acid</i> , butyric acid
25. CMP	Caproic acid

Note. The left column represents uv-absorbing compounds identified in liver extract. The right column gives compounds, tested up to now, which can be used as spacers. This table is only valid for the electrolyte system used in this work. The numeration refers to Figs. 1, 2, and 3.

^a The figures within parentheses give uv-absorbing maximum at 254 nm. The other substances are non-UV absorbing.

^b *Spacers* are of special interest because they increase the detectability where naturally occurring spacers in the liver extract are lacking or are in too low concentration.

and 360 nmol/g wet liver, respectively. ATP was spaced with tartaric acid and trichloroacetic acid to increase the separation on both sides of the peak. UDP is at the moment not separated from ATP because no spacer has been found.

The concentration of UDP was 90 nmol/g wet liver determined with hplc and subtracted from the ATP peak. The results are in good agreement with results of other authors (9–14).

CONCLUSIONS

Faupel *et al.* (9) have shown that stress, anoxia, and anesthesia change the nucleotide concentration of liver tissue. The extraction procedure is very critical due to lability of the nucleotides. The time until the tissue is frozen is kept short. By working in a cold room with cold chemicals and glassware the risk of high temperatures is reduced. The extraction has few steps

TABLE 3

RECOVERY OF ADDED ATP STANDARD AT DIFFERENT VOLUMES OF LIVER EXTRACT

Volume extract injected (μ l)	Volume ATP-std injected (μ l)	Measured amount of ATP (nmol)			Recovery of ATP-std (nmol)
		0.25% HPMC ($n = 3$)	0.50% HPMC ($n = 2$)	0.75% HPMC ($n = 2$)	
4.0	—	1.29	1.29	1.29	—
4.0	1.0	2.30	—	—	1.01
6.0	—	1.92	—	—	—
6.0	1.0	2.92	—	—	1.00
8.0	—	2.50	2.54	2.58	—
8.0	1.0	3.51	—	—	1.01

Note. The stabilizing medium HPMC, in the leading electrolyte, was examined at different concentrations. n = numbers of experiments.

minimizing metabolic changes, losses and saving time. The extract can be used directly without any freeze-drying or evaporation.

The use of isotachophoresis for separating and quantifying acid-extracted metabolites

from liver extracts is very valuable for uv-absorbing compounds. Mikkers *et al.* (15) have proposed a dual detection system where uv detection is combined with conductivity detection. This will evidently improve the reliability of isotachophoresis. Without a conductivity detector great care has to be taken and a comparison of uv absorption at 254 and 280 nm is recommended. Disadvantages compared to enzymatic and chromatographic methods are the quantitation of substances below 100 pmol and the lack of retention time, respectively. The latter makes applications on new fractions, e.g., on other tissues, time consuming because new careful identification is necessary. Isotachophoresis has a number of advantages: technical simplicity, unexpensive reagents, rapidity, minute injection volumes (about 10 μ l are needed), and ease in quantitation.

ACKNOWLEDGMENTS

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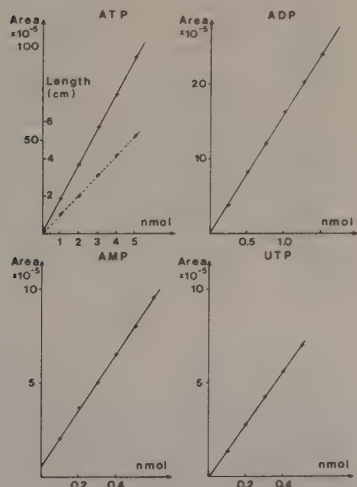


FIG. 5. Calibration curves for ATP, AMP, and UTP at 254 nm. The area is measured in $2 \times \text{mV} \times \text{ms}$, where the total potential difference of the signal is 89 mV. Area (—). Length (---).

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DECREASED UDP-GLUCURONIC ACID IN RAT LIVER AFTER ETHER NARCOSIS

An isotachophoretic study

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1. Introduction

For the past several years our laboratory has studied the effect of protein deficiency and cytostatics on the RNA metabolism of the liver in rat. With capillary isotachophoresis a fast screening method for determination of acid-extracted nucleotides was developed [1]. In that work unanaesthetized rats were killed by the double hatchet method [2] and the liver fragments obtained were rapidly frozen. This method seems to be the most accurate but we did not succeed in keeping the time from hatchet stroke to freeze-clamping [3] sufficiently short and constant. A sampling time of >7 s gave low nucleotide triphosphate concentrations and the ATP/ADP ratio dropped below 2.0.

Therefore, other methods of liver sampling, namely under diethylether or divinylether narcosis, were also tried primarily to obtain better reproducibility with high and constant ATP/ADP/AMP ratios. Unexpectedly these anaesthetic agents lowered the UDP-GlcUA level of the liver, a finding which prompted this publication.

2. Materials and methods

2.1. Chemicals

All nucleotides and substances for determining the UV peaks, β -alanine and *n*-caproic acid were purchased from Sigma Chemical Co. USA. Other materials were obtained as follows: HPMC, Dow Chemicals, USA; diethylether (Aether ad Narcosin), Skånska Bomulls-knutfabriken AB, Sweden and divinylether (Vinydan), H. Lundbeck, Denmark.

Abbreviations: UDP-GlcUA, UDP-glucuronic acid; HPMC, hydroxypropylmethylcellulose

2.2. Animals and liver sampling

Forty-six male Wistar rats (~150 g) were fed on a 25% casein diet [4] for 7 days. At about 8 a.m., 22 animals were anaesthetized in their cages placed inside a ventilated work bench. A funnel was put over the rat and oxygen was bubbled through the anaesthetic agent at 2 l/min. Diethylether (~4 ml) or 1.5 ml divinylether were necessary to achieve surgical anaesthesia. As soon as the rat was anaesthetized, it was transferred to a cork plate. In experiments where the rat was operated immediately, the anaesthetic administration was continued through a few layers of loosely fluffed gauze. The shortest time from the beginning of anaesthesia with diethylether and divinylether to sampling of liver tissue was 90 s and 60 s, respectively. For longer periods of anaesthesia the method with a plastic bag was used [5]. The abdomen was quickly opened, the left lobe of the liver was cut out with a pair of scissors and rapidly freeze-clamped between two metal blocks, precooled in liquid N_2 . The time from cutting to freezing was <1 s.

Liver sampling under narcosis was compared to the 8 rats of 24 killed with the double hatchet method [2] with the highest ATP/ADP ration (table 1). The sampling time for the 8 rats was 3–7 s [1].

2.3. Extraction and determination

The extraction with cold perchloric acid–methanol and neutralization with KOH were as in [1]. The quantitation was made with isotachophoresis on an LKB 2127 Tachophor (LKB, Sweden) equipped with a UV lamp at 254 nm. The separation was run in an 81 cm tube at 20°C. The electrolytes were 5 mM HCl + 0.25% HPMC + β -alanine to pH 3.89 and 10 mM caproic acid. Spacer solution [1] was used to increase separation. Additional to 5 μ l extract, 1.5 μ l following of the

spacer solution was added: 0.4 mM tartaric acid; 0.5 mM fumaric acid; 0.4 mM chloroacetic acid; 0.3 mM trichloroacetic acid; 0.2 mM glucaric acid; 0.3 mM glycolic acid; 0.3 mM glyceric acid; 0.5 mM gluconic acid; 0.3 mM acetic acid; 0.3 mM laevulinic acid; 0.8 mM propionic acid. The peaks were monitored with an LDC 304-50 computing integrator (Laboratory Data Control, England).

2.4. Calibration

UTP, GTP, ATP and CTP were calibrated simultaneously in concentrations normally occurring in rat liver. Phosphoenol pyruvic acid (0.25 nmol) was added as spacer between UTP and GTP, and trichloroacetic acid (0.50 nmol) between ATP and CTP. All calibration curves had correlation coefficients of 0.9985 or better. UDP-GlcUA was also calibrated with a correlation coefficient of 0.9999.

3. Results and discussion

Fig.1 shows two scans from liver extracts, sampled with the double hatchet method and under diethyl-

ether narcosis. The ATP/ADP/AMP ratios are equal and high (15/4/1) indicating good methodologic conditions. The striking difference between the two scans is the heavy decrease of UDP-GlcUA for the sample collected under diethylether narcosis. Fig.2 shows the relation between length of anaesthesia and amount of UDP-GlcUA. Diethylether decreased the UDP-GlcUA in <90 s and the pool was 20% of normal after 5 min anaesthesia. Divinylether, which gives quicker surgical anaesthesia, was also tested. The depression was not so pronounced, and 60 s anaesthesia gave almost normal values of UDP-GlcUA and UTP.

Workers using diethylether narcosis for liver sampling find low concentrations of UDP-GlcUA [6,7] compared to our results (table 1). With cervical dislocation values in the same range as ours are obtained [8,9].

One explanation for the decrease of the UDP-GlcUA pool might be that the products from the metabolism of ether [10,11] affect the pathway. Microsomal *O*-dealkylation [12] gives ethanol which can participate in glucuronidation [13,14] or change the NADH/NAD and NADPH/NADP ratios resulting in decreased UDP-

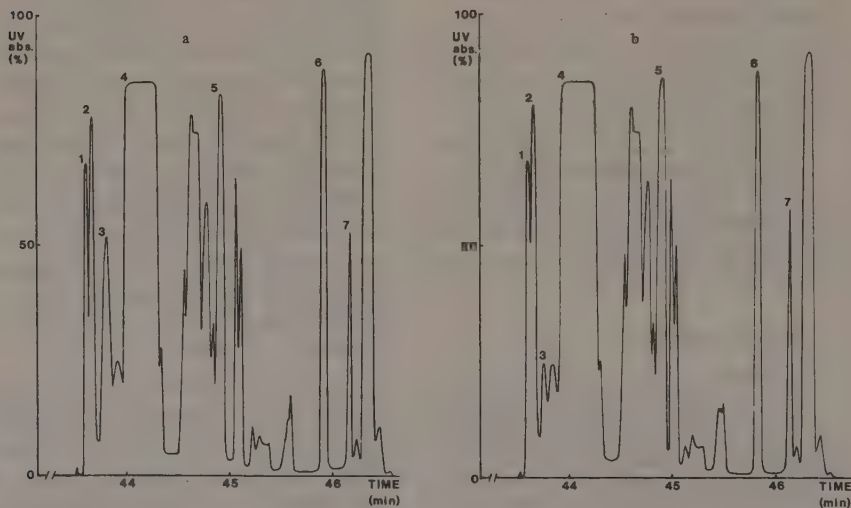


Fig.1. Isotachopheretic records from liver extracts sampled (a) with the double hatchet method and (b) under diethylether anaesthesia. Extract (5 μ l) with 1.5 μ l spacer solution was run in an isotachopheretic system and detected at 254 nm: (1) UTP; (2) GTP; (3) UDP-GlcUA; (4) ATP; (5) ADP; (6) ascorbic acid; (7) AMP. Other peaks in the records were described in [1].

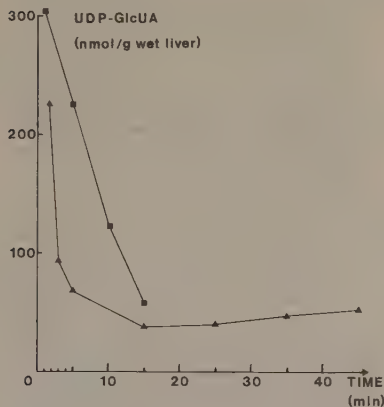


Fig.2. Decreased UDP-GlcUA (nmol/g wet liver) at extended anaesthesia with diethylether (▲) and divinylether (■). The low therapeutic index of divinylether made longer periods of anaesthesia difficult.

GlcUA formation and in increased flow of glucuronic acid to ascorbic acid [15].

A more likely explanation is a non-covalent regulatory mechanism [16], by which ether may affect the

microsomal membrane resulting in an increase of active enzyme sites. In [17] phospholipase A treatment of microsomes resulted in a 6-fold increase in the UDP-GlcUA transferase activity. Triton X-100 and other agents are also capable of influencing the protein-lipid interaction [18].

An induction of enzyme synthesis caused by exposure to diethylether [19] cannot explain our findings as the induction interval is too short. The production of free glucuronic acid via the pyrophosphatase-phosphatase route is another explanation but no glucuronic acid 1-phosphate was detected in the isotachogram.

In dogs anaesthetized for 2 h with diethylether, there was a 15-fold increase of ascorbic acid in liver urine over 24 h [20,21]. The following day the values were normal. The results from rats were somewhat lower. This agrees with our observation that the ascorbic acid pool in liver is elevated for animals anaesthetized 15–45 min compared to animals anaesthetized 90 s or animals in the double hatchet group ($0.001 < p < 0.01$).

In table 1 ribonucleotide concentrations are shown. The double hatchet method gave lower amounts of triphosphates probably because a sufficiently short time between killing and freezing (2.5–3.5 s) was difficult to achieve. In addition, there was no definite relation between sampling time and the ATP/ADP ratio. Factors as stress before killing and how and

Table 1
Nucleotide concentrations with different methods of liver sampling

	Double hatchet	Divinylether	Diethylether
Sampling time (s)	3–7	< 1	< 1
UTP	378 ± 7	379 ± 7	459 ± 12 ^a
GTP	421 ± 9 ^a	499 ± 5	464 ± 9 ^b
UDP-GlcUA	332 ± 20	322 ± 15	235 ± 13 ^b
ATP	2693 ± 46 ^a	3234 ± 46	3186 ± 23
ADP	1161 ± 47 ^a	919 ± 25	841 ± 29
AMP	348 ± 23 ^a	197 ± 16	258 ± 21
Σ (AMP, ADP, ATP)	4202 ± 66	4350 ± 54	4285 ± 39
Energy charge	0.779 ± 0.008 ^a	0.849 ± 0.005	0.842 ± 0.006
No. animals	8	6	6

^a Significance compared to the divinylether group; $p < 0.001$

^b Significance $0.001 < p < 0.01$

The shortest possible time of anaesthesia was used, that is 60–70 s and 90–100 s for the two agents, respectively. Values are given as means ± SEM (nmol/g wet liver tissue).

$$\text{Energy charge} = 0.5 \times \frac{2 \text{ ATP} + \text{ADP}}{(\text{ATP} + \text{ADP} + \text{AMP})} \quad [23]$$

where the hatchet stroke divided the liver may interfere. The advantages of anaesthesia were the constant and short sampling time (<1 s) and that the same liver lobe, the left lateral, was taken. This method gave higher amounts of nucleotide triphosphates and better reproducibility. Divinylether is an alternative for short operations. Its advantages over diethylether are a shorter anaesthesia induction time, a lesser influence on UDP-GlcUA and, consequently, less influence on UTP. Additionally, divinylether is less irritating for the respiratory tract and less excitatory.

Acknowledgements

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THE EFFECT OF 5-FLUOROOROTIC ACID ON THE EARLY LABELLING OF NUCLEOTIDES
AND RNA IN WHOLE LIVER AND OF RNA IN SUB-NUCLEAR FRACTIONS IN RATS GIVEN
³H-OROTIC ACID

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ABSTRACT

Male white rats were given either orotic acid or 5-fluoroorotic acid 60 min, and ³H-orotic acid 3 1/2 min before sacrifice. Liver nucleotides were analyzed by isotachopheresis. The (F)UTP pool increased to the same extent in both groups and showed the same specific labelling. The RNA synthesis, as measured by nmol of UTP entering RNA-UMP/g wet liver tissue was significantly lower in fluoroorotic acid treated rats. In the nucleolar fraction there was an increase in the RNA/DNA ratio, a decrease in the specific RNA labelling and an essentially unaltered specific labelling of RNA/μg DNA. The effect on the specific labelling of RNA/μg DNA was the same in the nucleolar fraction and the nucleolar-free pellet, suggesting that the transcription of RNA in these two fractions proceeded in a similar way. There was a slight increase in the specific RNA labelling of the supernatant sub-nuclear fraction, containing low-molecular weight RNA. Incorporation into UDP-hexose and UDP-N-acetylhexosamine was unchanged.

INTRODUCTION

We found that high doses of 5-fluorouracil enlarged the nucleoli in rat liver cells and altered their ultrastructure. They also depressed the RNA labelling of the liver after administration of a labelled precursor (ref.1). At low doses there was a delay or block in the maturation of pre-ribosomal RNA and no ribosomes or labelled ribosomal RNA appeared in the cytoplasm (refs.2,3). These observations have been greatly extended by several groups of workers and also include the effects of 5-fluoroorotic acid and similar fluoro compounds. The block in the nucleolar RNA

metabolism has generally been localized to the post-transcriptional processing steps (refs.4,5). There seems to be no impairment of either the labelling of heterogeneous (messenger) RNA (refs.6,7) or low-molecular weight RNA (refs.7,8). Transcription of nucleolar 45S RNA is essentially unaltered (ref.5), but may be depressed (ref.4). These studies have mainly been performed with labelled precursors, but the flow of labelling from, for example, orotic acid via UTP into both RNA, UDP-N-acetylhexosamine, UDP-hexose and UDP-glucuronic acid has not been analyzed and, therefore, the rate of RNA synthesis has been difficult to estimate. Isotachopheresis has made it possible to analyze these nucleotides in the liver (ref.9). Using ^3H -orotic acid we describe in this paper the effects of 5-fluoroorotic acid on the labelling of nucleotides and RNA in whole liver and of RNA in sub-nuclear fractions.

Animals

Male Wistar rats weighing about 180 g were fed on a 25% casein diet (ref.10) for 6 days and then starved overnight. At about 8 a.m., 60 min before sacrifice, the rats were given either 1500 nmol of orotic acid or 5-fluoroorotic acid per 100 g body weight, and 3 1/2 min before sacrifice 0.50 mCi per 100 g body weight ^3H -orotic acid by intraperitoneal injections. One min before liver sampling the rats were anaesthetized with divinylether (ref.11), and the abdomen was opened. The left lobe of the liver was freeze-clamped *in situ* and stored in liquid nitrogen for later extraction of nucleotides, RNA and DNA. The remaining part of the liver was used for preparation of sub-nuclear fractions.

Analysis of liver fractions

The freeze-clamped liver was extracted with perchloric acid and methanol as described previously (ref.9) with one modification. To maintain a low temperature the extraction was made in a cryostat (Linde, GFR) at -20 C. The neutralized supernatant was used for quantitative determination of the nucleotide pools and the incorporation of labelled orotic acid into the different uridine phosphates by isotachopheresis. The acid-insoluble pellet of the liver tissue was treated according to Munro and Fleck (ref.12) for determination of RNA and RNA labelling. The pellet used for

RNA determination was also used for an analysis of DNA by the technique by Scott et al. (ref.13) as modified by Hinrichs et al. (ref.14).

About 5 g of the excised liver was used for isolation of nuclei by a procedure based on the method of Chauveau et al (ref.15) and modified in our laboratory (refs.16,17). All procedures were performed at 0°C. The liver was rapidly passed through a plexiglass tissue press with 1 mm holes in the frontal plate and transferred to a 40 ml Dounce glass homogenizer (Kontes Glass Company, USA; clearance 0.0015-0.0025 inches), containing 4 ml 2.32 M sucrose with 0.013 M CaCl_2 per g pressed liver tissue, and homogenized with 13 up and down strokes in a cold room. 27 ml of homogenate was layered over 10 ml 2.32 M sucrose with 0.013 M CaCl_2 and centrifuged for 0.5 h at 112 000 g in a Sorvall OTD 2 ultracentrifuge AH-627 swing out rotor (Sorvall, USA). Aliquots of the original homogenate and the supernatant were frozen. The pellet containing the nuclei was suspended and homogenized in a 7 ml Dounce homogenizer with 10 ml 0.25 M sucrose and 0.003% potassium polyvinyl sulphate. One ml was frozen. To break the nuclei 9 ml of the homogenate was passed through a French press at 5 000 kg/cm² at a velocity of about 1 drop per second. Light microscopy revealed that more than 99.9% of the nuclei were broken. The first and the last drops, which may contain unbroken nuclei, were discarded. The remainder was layered over 3 ml of 1.1 M sucrose with 0.003% potassium polyvinyl phosphate, 0.025 M KCl, 0.01 M MgCl_2 in 0.05 M Tris-HCl buffer, pH 7.6, and centrifuged for 20 min at 2 000 g in a Sorvall RC2-B-centrifuge. The pellet, which contains most of the nucleoli, and the cushion were frozen separately. The supernatant was centrifuged once more over an identical cushion at 10 000 g for 20 min. The pellet and cushion were frozen together. The supernatant was centrifuged for 1 h at 25 400 g. The pellet, which was the nucleolar-free nuclear remnant, and the supernatant were frozen separately. The percentage of RNA in the nucleolar fraction, the nucleolar-free fraction and the supernatant was 8, 47 and 32, respectively, of total nuclear RNA. The percentage of DNA was 5, 56 and 17, respectively, of total nuclear DNA. The recovery of RNA and DNA was 85-95%. The base composition of the RNA fractions was determined by isotachopheresis after hydrolyzation for 17 h at 37°C with 0.3 M KOH (ref.18).

Isotachophoresis

The isotachophoresis was performed on an LKB 2127 Tachophor with a UV-detector at 254 nm as described previously (ref.9). Preparative isotachophoresis for radioactive determination was run in a Tachofrac kit (LKB, Sweden) described by Arlinger (ref.19). The recorder-paper and the fraction collection strip were run at 5 cm/min. The cellulose acetate strip was cut into 1 mm lengths in a stamp made in our laboratory (Fig.1). Each piece was put into a vial and 200 μ l glacial acetic acid was added to dissolve the cellulose. After two h with gentle shaking 5 ml Instafluor (Packard, USA) was added and scintillation countings were made in an SL 30 liquid scintillation spectrometer (Intertechnique, France).

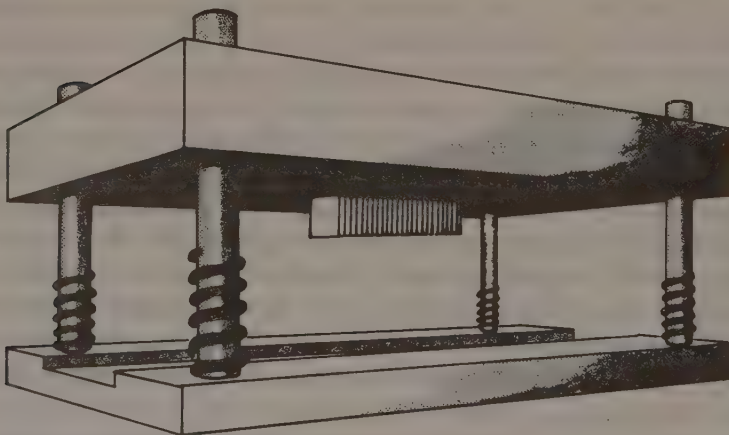


Fig. 1. The stamping apparatus. The fraction collection strip is taped to a thin cardboard so that tape covers just 1 mm of its width. This is put into the bottom groove. The knives on the top plate, positioned at 1 mm intervals, are pressed down onto the paper cutting it but not the tape. The 4 x 1 mm paper strips can be torn away from the tape with a pincett and put into the counting vials.

Gel electrophoresis

The RNA of the sub-nuclear fractions were extracted with phenol (ref.2)

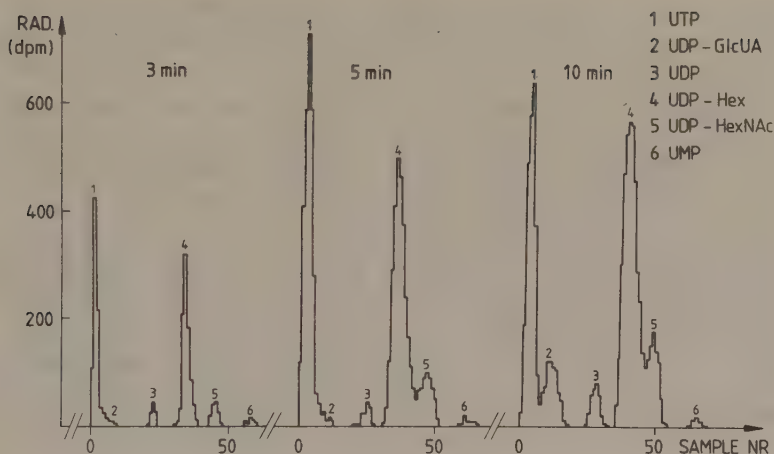


Fig. 2. Preliminary experiment. Tachofrac profiles of ^3H -nucleotide in labelling untreated rats following administration of ^3H -orotic acid.

for 20 min at 20°C and 85°C . The two extracts were combined. RNA was precipitated with ethanol, dissolved and submitted to gel electrophoresis (2.0% polyacrylamide, 0.5% agarose) for 50 min (ref.16). Optical density was recorded in a Joyce-Loeble (GB) gel scan 400 at 265 nm. After cutting the gel (ref.2) the discs were put into vials, covered with 0.2 ml N NaOH, kept at -20°C for 20 min, then at $+70^\circ\text{C}$ for 4 h and after cooling neutralized with 0.2 ml N HCl. 10 ml Instagel (Packard) was added. Scintillations were counted as described above.

Chemicals

The chemicals were purchased as follows: orotic acid, different spacers and nucleotides for calibrations from Sigma Chemical Co, USA; 5- ^3H -orotic acid (spec. act. 20 Ci/nmol) from the Radiochemical Centre, England; 5-fluoroorotic acid, kindly delivered by La Roche, Switzerland.

Preliminary experiments

Fig. 2 shows the incorporation profiles of ^3H -orotic acid after 3, 5

and 10 min labelling time. The transport and the uptake of orotic acid and the enzymatic reactions involved to convert it to UTP are very fast, and 3 min after the injection of ^3H -orotic acid all uridine phosphates were labelled. The low labelling of UMP and UDP indicates minimal breakdown of UTP and UDP-sugars during extraction. The results from incorporation profiles and from determination of the RNA labelling are plotted in Fig. 3. The incorporation was corrected for different uptake of orotic acid from the peritoneal cavity by dividing the labelling of the uridine nucleotides by the total labelling in the liver. The curves corresponded well to the simplified scheme of the anabolic pathway for orotic acid (Fig. 3), where UTP labelling increased rapidly followed by a heavy increase of UDP-hexose labelling and a delayed increase of UDP-glucuronic acid labelling. There was no detectable labelling of CTP. The specific activity of UTP at different labelling times is shown in Fig. 4. A la-

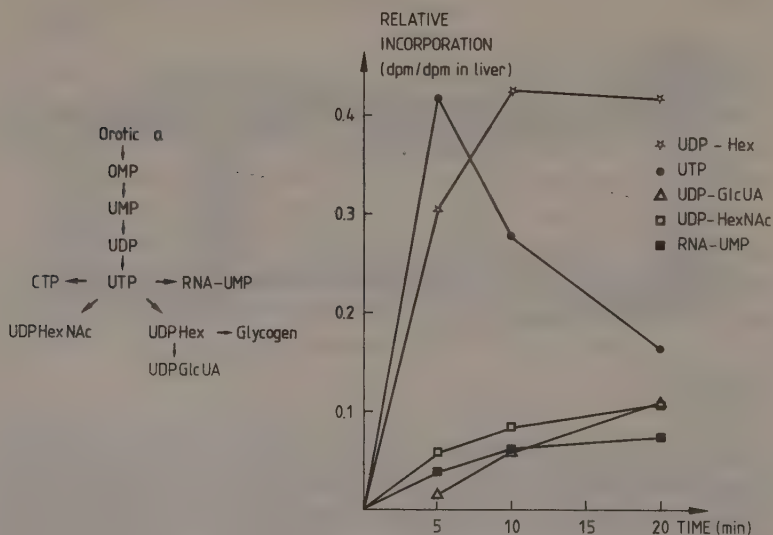


Fig. 3. Anabolism of ^3H -orotic acid. UDP-Hex = UDP-hexose, UDPHexNac = UDP-N-acetyl-hexosamine, UDPGlcUA = UDP-glucuronic acid.

bellling time of less than 5 min, when the specific activity of UTP is rising linearly, is necessary for calculation of RNA synthesis according to the formula used by Bucher and Swaffield (ref. 20), given in Fig. 5.

We chose a dose of 1500 nmol 5-fluoroorotic acid/100 g body weight, which is a relatively low dose (ref.6). To check the effect, rats were treated with this dose or, as a control, an equal dose of cold orotic acid for 60, 105 or 150 min. A tracer dose of ^3H -orotic acid was given 40 min before death. The labelling in the 40°C phenol extract of nuclear RNA (see ref. 18) decreased to 40%. In other rats given 5-fluoroorotic acid 150 min and ^3H -orotic acid 90 min before death, the labelling of the ribosomal fraction, prepared as in ref. 21, also decreased to 40% of that in controls. The dose was thus effective.

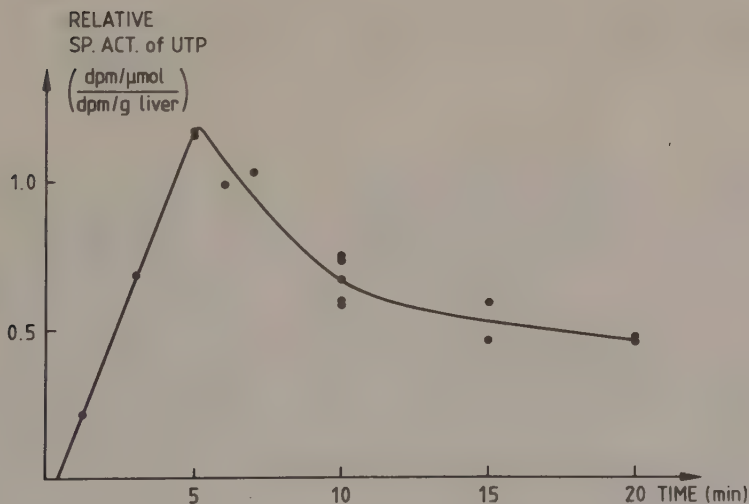


Fig. 4. Relative specific activity of UTP after administration of ^3H -orotic acid.

RESULTS AND DISCUSSION

Administration of uridine precursors increased the UTP-pool. The doses of orotic acid and 5-fluoroorotic acid given in this experiment increased the UTP-pool to the same extent compared to rats not given any precursor, namely from about 325 to 400 nmol/g wet liver tissue (125 to 150 nmol/mg DNA) (Fig. 5). The specific activity of UTP, calculated as dpm/nmol UTP, varied considerably from rat to rat, from 3 800 to 16 000. These differences can be explained by the instability of 5-³H-orotic acid (self-radiolysis) and by fluctuations in uptake from the peritoneal cavity. ³H-orotic acid was, however, preferred to ¹⁴C-orotic acid due to its higher specific activity. After compensation for those variables by dividing the specific activity of UTP with the total activity in g liver, we found no differences between the two groups (t=0.61). The flow of UTP to UDP-hexose and UDP-N-acetylhexoseamine was not significantly different between the two groups. The labelling time was too short for adequate detection of activity in UDP-glucuronic acid (Fig.2).

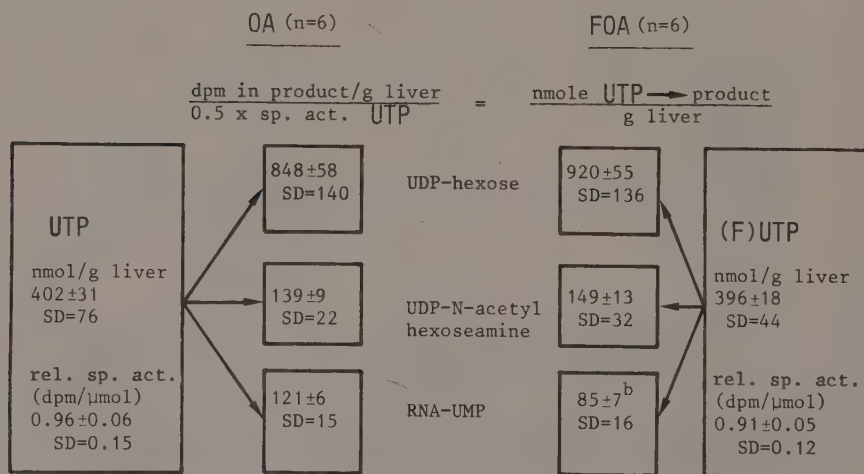


Fig. 5. Labelling of nucleotides and RNA in whole liver.

The synthesis of RNA extracted from total liver as measured by nmol of UTP entering RNA-UMP/g wet liver was significantly lower in fluoroarotic acid treated rats ($t=4.02$). The same decrease, namely 28% ($t=3.22$, $n=20$), was obtained in a similar experiment using a 5 min labelling time and without overnight starvation. The hypothesized decrease in RNA synthesis implies that the figures of the UTP-pool of the extract of total liver reflect the conditions at the transcription site. Siebert and co-workers (ref.22) have shown with nonaqueous isolation technique that different nucleotides are present at the same concentrations in the nuclei and the cytoplasm in rat liver, and that the labelling is "near-identical". A difference in amount and specific labelling of UTP and (F)UTP in the nucleus, due to the greater electronegativity of FUTP, given a lower pKa and higher degree of dissociation than UTP, cannot, however, be excluded. It is our intention to adapt nonaqueous isolation technique for further studies of these problems.

	$\frac{\text{sp. act. RNA}}{\text{sp. act. nRNA}} \times 100$		$\frac{\text{RNA}}{\text{DNA}} \times 100$		$\frac{\text{sp. act. RNA}}{\text{sp. act. nRNA}} \times \frac{\text{RNA}}{\text{DNA}} \times 100$	
	OA	FOA	OA	FOA	OA	FOA
Nucleolar fraction	99 $\pm$ 2 SD=5	67 $\pm$ 3 ^c SD=7	20.0 $\pm$ 0.3 SD=0.7	27.2 $\pm$ 1.3 ^c SD=3.3	20.0 $\pm$ 0.6 SD=1.3	18.0 $\pm$ 0.3 ^a SD=0.7
Nucleolar-free fraction	91 $\pm$ 2 SD=4	88 $\pm$ 2 SD=6	14.1 $\pm$ 0.3 SD=0.7	11.6 $\pm$ 0.5 ^b SD=1.2	12.8 $\pm$ 0.4 SD=0.9	10.3 $\pm$ 0.6 ^b SD=1.4
Supernatant	99 $\pm$ 1 SD=3	117 $\pm$ 2 ^c SD=6	29.0 $\pm$ 1.7 SD=3.0	28.2 $\pm$ 2.6 SD=6.6	28.6 $\pm$ 2.3 SD=4.0	33.1 $\pm$ 3.6 SD=8.9
Number of rats	5	6	5	6	5	6

^a0.02>p>0.01

^b0.01>p>0.001

^c0.001>p

Table 1. RNA and DNA of sub-nuclear fractions.

Gel electrophoresis of sub-nuclear fractions revealed that the supernatant contained low-molecular weight RNA and the nucleolar-free pellet heterogeneous RNA.

The (G+C)/(U+A) base ratios were 1.18, 1.56 and 1.26, with no differences between the groups, in the supernatant, the nucleolar fraction and the nucleolar-free pellet, respectively.

A main finding was the substantial increase in the RNA/DNA ratio of the nucleolar fraction in the fluoroorotic acid-treated rats (Table 1), in agreement with our demonstration of enlarged nucleoli at treatment with 5-fluorouracil (refs.1,3). There was a concomitant decrease in the specific nucleolar RNA labelling, resulting in an essentially unaltered specific labelling of RNA/ μ g DNA in the nucleolar fraction. The effect of fluoroorotic acid on the specific labelling of RNA/ μ g DNA was the same in the nucleolar fraction and in the nucleolar-free pellet, containing heterogeneous RNA, suggesting that the transcription of RNA in these two fractions proceeded in a similar way. As discussed above, our results on total liver RNA indicated a decreased synthesis.

There was a slight increase in the specific RNA labelling of the supernatant sub-nuclear fraction, which by gel electrophoresis was shown to contain low-molecular weight RNA. The synthesis of this RNA has been reported to be normal following treatment by fluoro compounds (refs.7,8). An interesting possibility, suggested by observations on Miller preparations of fluorouridine treated specimens (ref. 23), is that false RNA may loosen prematurely from the DNA thread and, possibly, accumulate in the supernatant fraction, altering its specific labelling.

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ISOTACHOPHORETIC DETERMINATION OF THE BASE COMPOSITION IN RAT LIVER RNA WITH AND WITHOUT A COLUMN COUPLING SYSTEM ADAPTED TO A TACHOPHOR CAPILLARY

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ABSTRACT

Following extraction of acid-soluble nucleotides with cold perchloric acid, RNA from whole liver was extracted and hydrolyzed with 0.3 M KOH. Nuclear RNA was extracted from a nuclear pellet with phenol at 0°C, 40°C, 51°C and 85°C and hydrolyzed. The 2', 3'-monophosphates were determined by isotachophoresis. The leading electrolyte system was buffered to pH 4.06 and run with or without a column coupling system adapted to a Tachophor capillary. Only 0.20 µg hydrolyzed RNA was needed to determine the amount of the four 2',3'-monophosphates and the base composition in different RNA fractions.

INTRODUCTION

The methods widely employed for determination of the base composition of RNA are based on separation and spectrophotometric measurement of the four 2',3'-ribonucleotides formed after alkaline hydrolysis of RNA. The four nucleotides have been separated by paper electrophoresis (ref.1), paper chromatography (ref.2), column chromatography on strong anion exchange resins (refs.3,4) or high pressure liquid chromatography (HPLC) (ref.5). Paper electrophoresis requires concentrated solutions and is tedious. Large volumes are needed to elute GMP and UMP by column chromatography. HPLC has the disadvantages of column packing and long regeneration times

Isotachophoresis has been used in our laboratory as a rapid method for separation of acid-soluble nucleotides in rat liver (ref.6). The present paper will describe how this technique can be employed to determine the base composition of various RNA fractions.

MATERIALS AND METHODS

Animals

Wistar rats were fed on a 25% casein diet (ref.7) for 7 days to a final weight of 200 g, approximately.

Extraction

In studies on the base composition of RNA in whole liver tissue, the livers were freeze-clamped in situ with a pair of tongs precooled in liquid nitrogen. The tissue was extracted in 0.7 M perchloric acid with 20% methanol at -20°C (Fig. 1, ref.6). The pellet was washed twice

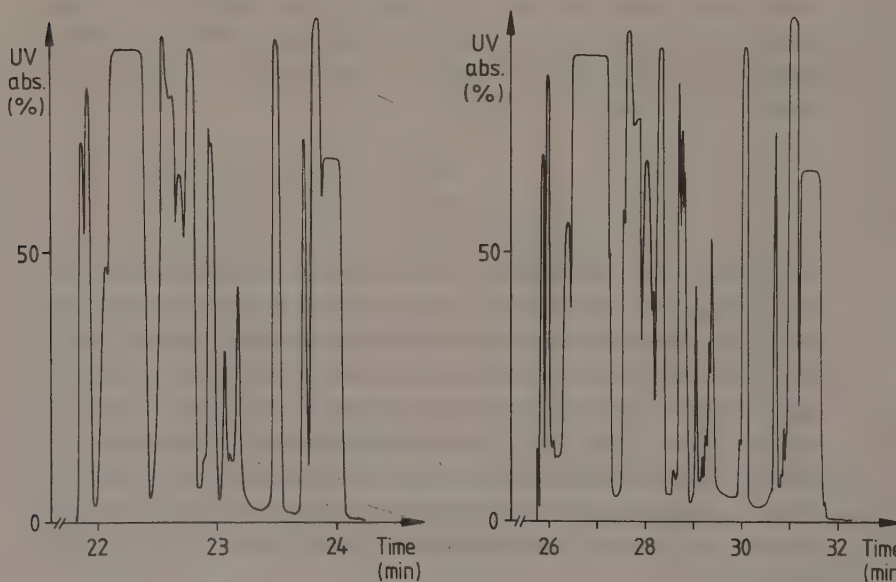


Fig. 1. Two records of a nucleotide extract from rat liver without external spacers. a) 4 μl extract, a maximum volume without overloading the system, injected in an ordinary 43 cm Tachophor capillary. Recorder speed: 5 cm/min. b) 10 μl extract injected in a column coupling system. Recorder speed: 2 cm/min.

with 0.2 M perchloric acid with 15% methanol, homogenized in 40 ml 0.3 M KOH per g wet liver and hydrolyzed at 37°C. Three ml of the sample was removed after one h and then after every fourth h during the hydrolyzation up to 25 h. These three ml samples were each treated with 1.875 ml 1.2 perchloric acid in 52% methanol. After 10 min centrifugation at -10°C and 7,500 g, 4 ml of the supernatant was added to 200 µl of 0.4 M triethanolamine buffer at pH 7.2 and 100 µl 10 M KOH. The sample was adjusted to pH 7.0 with 2 M KOH.

In studies of nuclear RNA, a liver homogenate was prepared and the nuclei pelleted by ultracentrifugation according to Dabeva et al (ref.8). The nuclear pellet from 6 g of liver was extracted twice at 0°C for 5 min and once at 40°C, 51°C and 85°C for 10min in 0.01 M Na-acetate-buffer, pH 5.6, with 0.14 M NaCl and an equal part freshly prepared phenol with 0.1% 8-OH-quinoline. For the extraction at 85°C, sodiumdodecylsulphate was added to the buffer to a final concentration of 0.5%. After one phenol and one chloroform washing the RNA was precipitated with ethanol according to Shapiro (ref.9). The pellet was hydrolyzed for 17 h at 37°C with 1 ml 0.3 M KOH. One ml of the nuclear RNA preparations was treated in the same way and with the same proportions as those from whole liver RNA.

The neutralized solutions were then ready for analysis with isotachopheresis.

Isotachopheresis

The separation was performed on an LKB 2127 Tachophor (LKB, Sweden) equipped with a UV lamp at 254 nm either with a 43 cm capillary or a column coupling system adapted for a LKB Tachophor.

The leading system finally chosen for the 43 cm capillary was 5 mM HCl with 0.25% hydroxypropylmethylcellulose (HPMC) and 0.2 M β -alanine to reach a pH of 4.06. The terminating electrolyte was 10 mM caproic acid. Another leading electrolyte system, which gave just as good results, was the same electrolyte buffered to pH 3.80. With this system aspartic acid was needed to space UMP from GMP. α -aminobutyric acid (GABA) was also

tested as a counter ion but the separation was not satisfying in the pH range 3.80 - 4.15.

The column coupling system shown in Fig. 2 was constructed as described by Verheggen et al (ref.10). A 43 cm capillary was shortened at the right side to make room for the column coupling block consisting of the bifurcation and the preseparation channel. The bifurcation was dimensioned as described by Verheggen et al (ref.10) but the preseparation channel was made shorter and wider (33 mm with $d_i=2$ mm and 14.5 mm with $d_i=1$ mm). The conductivity detector with a 1000 Hz sine wave oscillator and an operational amplifier has two platina electrodes placed 2.5 and 3.5 mm in front of the bifurcation. The electrolyte system used for column coupling was the same as for the 43 cm capillary except that the concentration of HPMC was 0.6% in the leading preseparation channel and the terminating channel. This was necessary to stabilize the zones in the wide preseparation channel. The run was started at 325 μ A, and when the conductivity decreased after about 7 min the current was reduced to 75 μ A. At about 8 min the conductivity dropped sharply, and with a stop watch the 40 s delay-time was measured. After the switch to final separation, the current was increased to 175 μ A to shorten the separation time. The maximum voltage allowed was 12.5 kV and therefore the current was reduced as follows: 175 - 125 - 75 - 37 μ A. The first boundary appeared after approximately 25 min and at 9 kV (37 μ A).

The UV signals were monitored with an LDC 304 - 50 computing integrator (Laboratory Data Control, England) (ref.6).

Calibration

The calibration curves were made for both the 2'- and 3'-isomers and the mixed 2', 3'-isomers of the four monophosphates (U, G, A, C) after hydrolysis, precipitation and neutralization in the same way as for the liver samples. The four monophosphates were calibrated simultaneously (Fig. 3) and in the same range as in those present in the liver samples. The mixed isomers gave the same linearity as the eight 2' and 3'-monophosphates. The correlation coefficient was 0.9995 or better.

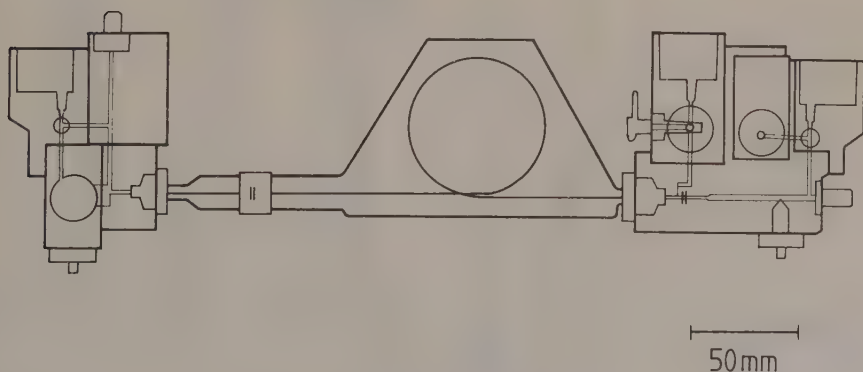
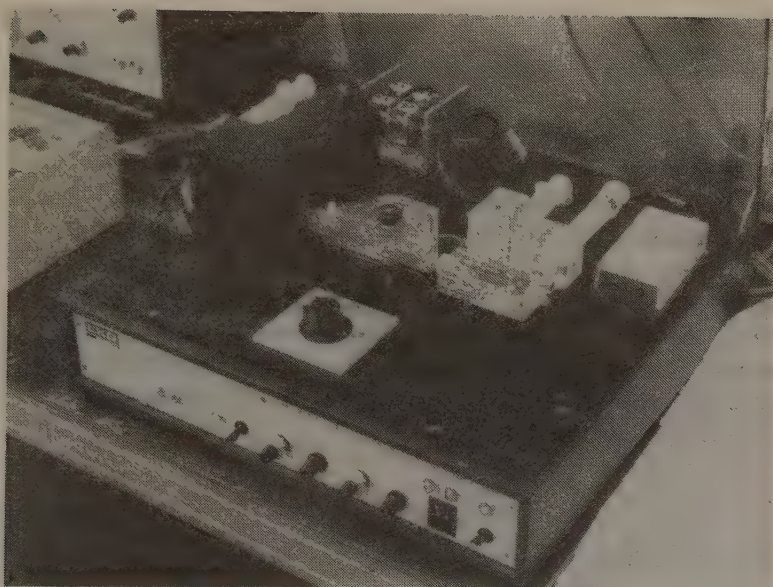


Fig. 2. The column coupling system mounted on an LKB Tachophor. In the upper picture the system is shown with the conductivity detector (white box to the right) and the high voltage relay (behind the UV-detector).

Chemicals

The 2'- and 3'-isomers and the mixed 2',3'-isomers of the four mono-phosphates (U, G, A, C), β -alanine and caproic acid were purchased from Sigma Chemicals Co., USA, and used without further purification. HPMC from Dow Chemical Co., USA, was dissolved in water treated with a milli-Q system (Millipore Corp., USA) in a cold room at 0.5% or 1% (w/v). After 24 h the solution was filtered under pressure through an AP 20 filter with an AP 25 prefilter (Millipore Corp., USA) and was ion exchanged in Amberlite MB 1 (Mallinckrodt Chem. Works, USA). The stock solution was kept at 4°C. Other chemicals were purchased as follows: phenol, 8-OH-quinoline and chloroform, Merck, FRG; sodiumdodecylsulphate, KEBO AB, Sweden. The phenol was glass-distilled before use. All chemicals used were pro analysi.

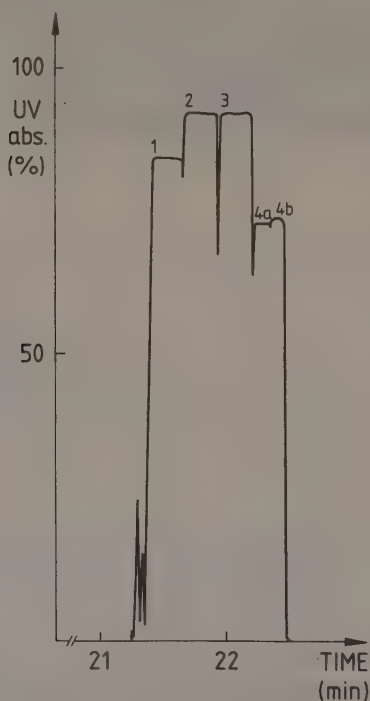


Fig. 3. Isotachophoretic recorder at 254 nm of the eight standard monophosphates. Injection volume: 8 μ l. Concentration: 1 nmole/ μ l of each monophosphate. (1) U-2',3'-MP; (2) G-2',3'-MP; (3) A-2',3'-MP; (4a) C-3'-MP; (4b) C-2'-MP.

RESULTS AND DISCUSSION

Fig. 4 shows whole liver RNA hydrolyzed for different time periods. After one h the four monophosphates are detectable but the oligonucleotides, some of which are seen in front of the quartet, make up the main part of the sample. After 9 and 17 h the oligonucleotides have disappeared and the monophosphates increased. In Fig. 5 the relation between amount of monophosphates and time of hydrolysis is shown. After about 10 h the amount of the four monophosphates has reached a plateau and therefore the usual time of hydrolysis, 17 h, is a well chosen time. As seen in Fig. 5, the base composition is typical for ribosomes, in agreement with the dominance of ribosomal RNA in the total liver with a $(G+C)/(U+A)$ ratio of 1.64. The amount of RNA calculated from the four monophosphates is about 8 300 $\mu\text{g/g}$ wet liver. A conventional hydrolysis

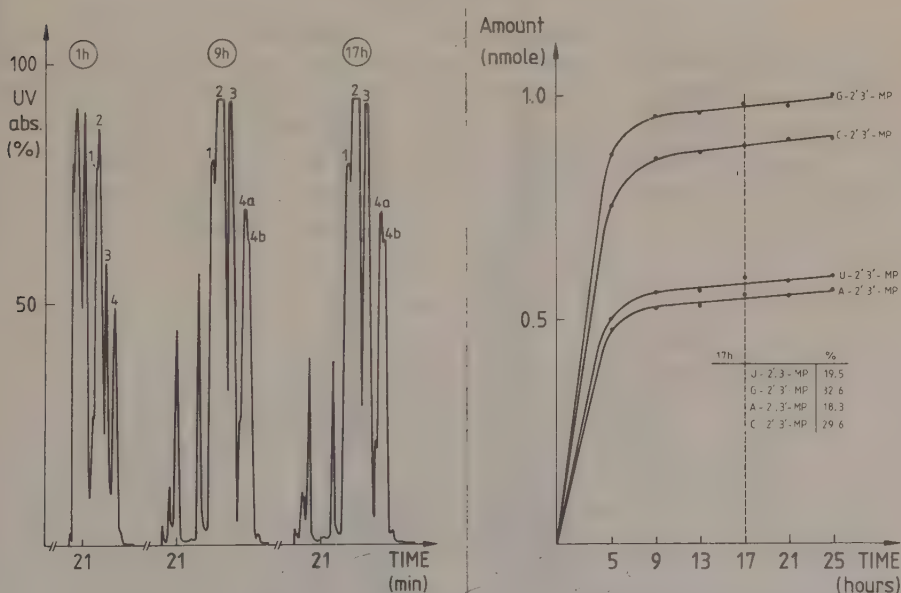


Fig. 4. Three records of whole liver RNA hydrolyzed at three different times. Injection volume: 5 μl . Numeration refers to Fig. 1.

Fig. 5. The relation between amount of hydrolyzed RNA from whole liver tissue and time of hydrolysis. The base composition at the usual time of extraction is inserted.

of 1 h according to Munro and Fleck (ref.12), where 32 μg RNA/ml correspond to an extinction of 1.000 at 260 nm, gave a result of 8 000 μg RNA/g wet liver.

Fig. 6 shows the records from the nuclear RNA extracted with phenol at 40°C and 85°C, where the column coupling system has been used. The optical density at 260 nm was 0.25–0.35. With volumes not overloading the system, the peaks were very near the detection limit when using a 43 cm Tachophor capillary. To be able to measure the peaks quantitatively, the sample can be freeze-dried and dissolved in a small volume, giving a higher concentration of the ribonucleotides without dissolving all the potassium perchlorate. With the column coupling system, 20 μl of the

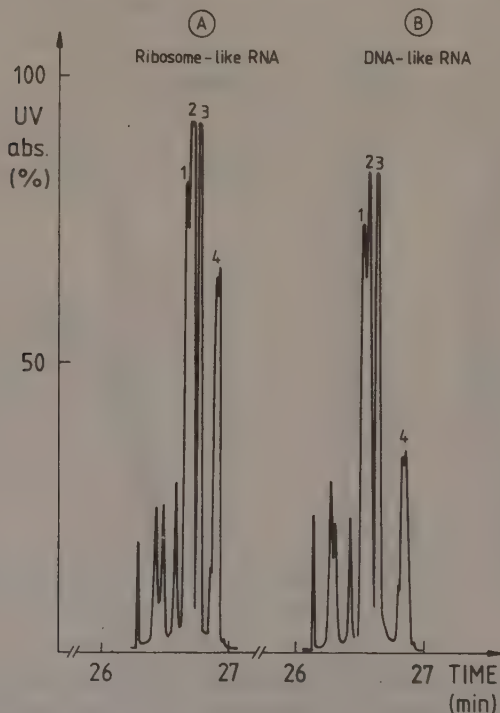


Fig. 6. Two isotachophoretic records from nuclear RNA extracted with phenol and hydrolyzed. Column coupling was used and 20 μl of each sample was injected. Numeration refers to Fig. 1.

original sample can be injected and the peaks easily measured. The 40°C -fraction is mainly RNA of ribosomal type with a $(\text{G}+\text{C})/(\text{U}+\text{A})$ ratio of 1.64. Base composition: U 20.5%; G 31.3%; A 17.2%; C 30.9%. The 85°C -fraction has a $(\text{G}+\text{C})/(\text{U}+\text{A})$ ratio of 0.75 which means that relatively pure DNA-like RNA is extracted at this temperature. Base composition: U 29.9%; G 21.9%; A 27.1%; C 21.1%. The 51°C -fraction (not shown) consists of 50% ribosome-like RNA and 50% DNA-like RNA, approximately. The $(\text{G}+\text{C})/(\text{U}+\text{A})$ ratio is around 1.05.

This study demonstrates that isotachopheresis is a simple and rapid method for separation and determination of 2',3'-ribonucleotides and that column coupling adapted to a Tachophor capillary extend the range where isotachopheresis can be used.

ACKNOWLEDGEMENTS

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EFFECTS OF γ -RADIATION AND HYPERTHERMIA ON DNA REPAIR SYNTHESIS AND THE LEVEL OF NAD^+ IN CULTURED HUMAN MONONUCLEAR LEUKOCYTES.

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ABSTRACT

We have investigated DNA repair, estimated by unscheduled DNA synthesis (UDS) and the cellular NAD^+ pool, after exposing human mononuclear leukocytes to hyperthermia and γ -radiation separately and in combination. We found that γ -ray induced a UDS decline with increasing temperature through the temperature region studied, 37⁰-45⁰C. At 42.5⁰C the γ -ray induced UDS was reduced to about 70% of the value found at 37⁰C. Following γ -ray damage the NAD^+ pool dropped to about 20% of control values. Without hyperthermic treatment the cells completely recovered to the original level within 5 h. Moderate hyperthermia (42.5⁰C for 45 min) followed by γ -ray damage altered the kinetics so that even after 8 h the NAD^+ pool had only recovered to 70% of the original level. After heat treatment at 44⁰C for 45 min prior to γ -radiation the cells did not recover at all, presumably because of the cytotoxic effects from the combined treatment.

INTRODUCTION

Substantial evidence for cytotoxic effects on tumor cells after a combined treatment with hyperthermia and ionizing radiation has accumulated (1-4) and such a treatment is becoming a potential useful tool in cancer therapy.

It is known that well oxygenated cells or tissues are much more sensitive to ionizing radiation than when they are hypoxic (5-7). In living tissue hyperthermia may induce physiological modifications. Bicher et

al (8) have found at 41°C an increased tissue oxygen tension following increased micro blood flow, thereby increasing the radiosensitivity of tumors. At higher temperatures the micro blood flow eventually collapses resulting in lower oxygen tension and further reduction of the already low tumor pH which, by itself, may be more lethal for tumor than for normal tissue.

Furthermore it is known that tissues which have a large portion of their cells in the DNA synthetic phase of the cell cycle (e.g. tumor cells) are more sensitive to hyperthermia than tissues with cells in other phases (9-12).

Taken together these data support the hypothesis that hyperthermia in combination with ionizing radiation may be used to selectively destroy neoplastic cells while sparing the less sensitive normal tissue.

One effect of hyperthermia when combined with DNA damaging agents is a decreased rate of repair of DNA strand breaks (13-20). The molecular basis of these findings is by no means clear and several hypotheses have been suggested: (i) increased efficiency of production of DNA damage, (ii) inhibition of enzymatic repair, (iii) partial denaturation of DNA, histones or other nucleoproteins, (iv) effects on RNA metabolism, (v) altered cellular membranes, (vi) effects on lysosomal activity, etc (see review by Roti Roti (21)).

Warters and Roti Roti (17,20) have suggested that changes in the chromatin structure, rather than enzyme denaturation, reduce the repair rate in γ -irradiated cells after hyperthermic exposure above 43°C. There is at least one enzyme which is known to alter chromatin structure during repair, namely poly(ADP-ribose)polymerase. This chromosomal enzyme catalyzes the polymerization of ADP-ribose moieties derived from NAD^+ to chromatin proteins including histones (22). Several studies now indicate that this enzyme has a role in cellular repair of DNA damage (23-34).

Cytotoxic agents are known to induce the activity of poly(ADP-ribose)polymerase thereby depleting the NAD^+ pool which, however, later recover (25). The apparent role of NAD^+ in cell metabolism and DNA repair has stimulated us to look for a possible effect of hyperthermia on the cel-

lular NAD^+ pool in connection to DNA damage induced by γ -radiation.

In this paper we have investigated unscheduled DNA synthesis (UDS), cellular NAD^+ levels, ATP, ADP, and AMP pools after exposing human mononuclear leukocytes to hyperthermia and γ -radiation separately and in combination. Our results indicate that hyperthermia and γ -radiation interact synergistically by inhibiting the recovery of depleted cellular NAD^+ levels.

METHODS AND MATERIALS

Isolation of mononuclear leukocytes. Human venous blood was collected in heparinized vacutainers (143 U.S.P. units/ tube). The platelet rich plasma was removed by centrifugation at 100 g for 10 min and physiologic saline solution was added to the original volume. 30 ml of the suspension was carefully layered on 15 ml Isopaque-Ficoll solution (Lymphoprep Nyegaard and Co.). After centrifugation at 400 g for 30 min the mononuclear blood cells were collected from the interphase, and the plasma liberated from the platelets. The mononuclear leukocytes were washed in saline solution and counted, then resuspended in Eagle's MEM with Hank's salts (GIBCO). More than 90% of the platelets are removed when the cells are prepared in this manner. Cultures contained about 15×10^6 cells in 4 ml of this medium plus 1 ml autologous plasma, 2.5 mM L-glutamine, penicillin (50 $\mu\text{g}/\text{ml}$) and streptomycin (50 $\mu\text{g}/\text{ml}$). The preparation of cultures was done at room temperature.

Hyperthermia treatment and γ -irradiation. In all experiments hyperthermia was given prior to radiation. The cells were placed for 45 min in waterbaths with temperatures varying from 39.5°C to 45°C and in parallel to control samples at 37°C for the same time. The temperatures were measured with an accuracy of about $\pm 0.2^\circ\text{C}$. The heat treatment period of 45 min was chosen because that time is used in clinical application of hyperthermia and radiation in our hospital (4).

Immediately after the hyperthermia treatment the cell cultures were put on ice to retard the DNA repair process and irradiated in a ^{137}Cs γ -ray source (Scandiatronix "Gammarad 900", 0.8 Gy/min) parallel to

controls on ice.

After the irradiation the samples for UDS-measurements were incubated for 18 h at 37°C. Samples for NAD⁺ pool measurements were incubated for various times from 15 min up to 8 h at 37°C. Pilot experiments showed that the samples equilibrated to the desired temperature within 30 min.

Measurements of UDS. For the radiation-induced repair synthesis measurements 10 mM hydroxyurea (Sigma) was added immediately after the preparation of the cultures and ³H-thymidine (10 µCi/ml, 25 Ci/mmol, Amersham) was added immediately after the irradiation step. Hydroxyurea is used to suppress incorporation of ³H-thymidine due to ordinary replicative DNA synthesis. After 18 h repair time the cells were harvested by centrifugation at 300 g for 10 min. The DNA was extracted, purified, radioactivity counted and quantitated by the method described previously (35). The results were recorded as cpm ³H-dThd incorporated per µg DNA.

Measurement of NAD⁺, ATP, ADP, and AMP pools. After the appropriate incubation times the cells were harvested by centrifugation for 5 min at 300 g and +20°C. Each sample was suspended in 1.8 ml physiologic saline and transferred to 2 ml glass homogenizers and 25 µl aliquots were taken for manual cell counting. The cells were spun down for 30 s at 2000 g and +4°C, and the supernatant was discarded. The pellets in the homogenizers were frozen in liquid nitrogen and stored at -80°C.

The day after preparation the homogenizers were brought to a cryostat (Linde) cooled to -20°C. To the frozen pellets 10 µl of a solution composed of 0.70 M perchloric acid, 30% (v/v) methanol and 0.5 mM nicotinic acid were added per 10⁶ cells. The pellets were loosened and homogenized for 3 min. The homogenizers with the pestles were centrifuged for 30 s at 150 g and -20°C. The pestles were removed and the extracts were centrifuged again for 1 min at 12000 g. The tubes were put into the cryostat. Aliquots of the supernatants were transferred to 1.5 ml Eppendorf tubes containing a mixture of 150 µl 7 M KOH, 200 µl 0.4 M triethanolamine buffered with HCl to pH 7.2 and 50 µl thymol blue (3 mg/10 ml 8 mM KOH). The proportion between the mixture and the aliquot

of the supernatant was 1:5. The tubes were stirred, 2 M KOH was added until the color changed from red to yellow, and then the solution was carefully adjusted to pH 6.6-7.0 as determined with indicator paper (pH-range 6.4-8.0, Merck). The extracts were then centrifuged for 1 min at 44000 g and -20°C , and the supernatants were transferred to Eppendorf tubes and stored at -80°C .

Isotachophoresis was performed on an LKB 2127 Tachophor with an electrolyte system as described previously (36). To 4 μl cell extract, 1 μl of the following spacer solution was used to increase separation: 0.3 mM phosphoenolpyruvic acid, 0.3 mM phosphoglyceric acid, 0.4 mM chloroacetic acid, 0.3 mM trichloroacetic acid, 0.05 mM CTP, 0.3 mM glucaric acid, 0.4 mM glyceric acid, 0.3 mM acetic acid, 0.4 mM laevulinic acid and 0.8 mM propionic acid. Nicotinic acid added at the extraction was used as an internal standard to calculate the dilution constant. Concentration of different substances could then be determined per 10^6 cells.

Estimation of viabilities. After the appropriate incubation times aliquots were taken out and mixed with an equal volume of 0.4% trypan blue dissolved in Ringer's solution. The cell suspensions were incubated at 37°C for 30 min and immediately placed on ice. The percentage of unstained (viable) cells was then determined by light microscopy.

RESULTS

γ -ray induced UDS and hyperthermia. The data in Fig. 1 show the level of UDS following damage of the mononuclear leukocytes by 30 Gy γ -rays versus temperature with the hyperthermic treatment immediately before the irradiation. All data points were obtained with cells from the same individual. UDS declined with increasing temperature as evidenced by a highly significant linear regression correlation coefficient between temperature and UDS of $r=-0.91$ ($p<0.001$). At 42.5°C the UDS level was about 30% lower than at the control temperature of 37°C .

The level of hydroxyurea suppressed replicative synthesis showed a slight decrease with increasing temperature, which, however, was not

statistically significant.

NAD⁺ pools after γ -radiation and hyperthermia. A typical nucleotide profile is given in Fig. 2. The various nucleotides were identified using the same criteria as already described in detail (36). We have utilized freshly isolated mononuclear leukocytes from 18 blood donors. The NAD⁺ levels varied for these individuals from 78 to 144 pmol/10⁶ cells with an average value of 118 ± 17 pmol/10⁶ cells (mean $\pm$ S.D.). Berger *et al* (37) found for 20 blood donors NAD⁺ levels between 40 and 114 pmol/10⁶ cells.

Figure 3 shows NAD⁺ levels versus incubation time at 37°C following 30 Gy radiation exposure and hyperthermia treatment for 45 min at 37°C (controls), 42.5°C and 44°C prior to radiation. Hyperthermia exposure itself did not alter the NAD⁺ pool outside the reproducibility range of our method, being about $\pm 5\%$. However, cells treated at 44 °C showed a slight reduction of the NAD⁺ level for incubation times longer than 5 h (data not shown).

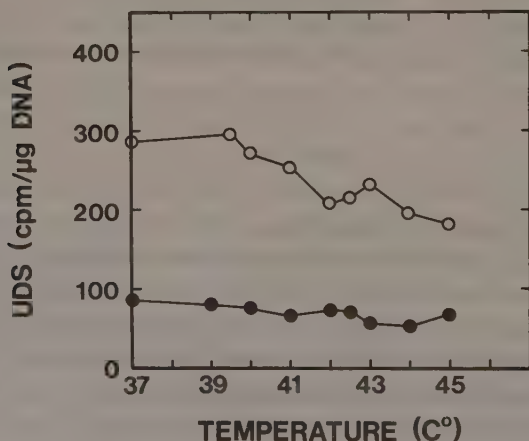


Fig. 1. Unscheduled incorporation of ³H-dThd following DNA-damage by 30 Gy γ -radiation as a function of heat treatments for 45 min given immediately prior to the radiation (open circles). Filled circles show the corresponding hydroxyurea-suppressed replicative synthesis.

Damaging the mononuclear leukocytes with γ -radiation dramatically depleted the NAD^+ pool to less than 20% of the original value. The NAD^+ pool was completely restored within 5 h when the cells were incubated at the control temperature of 37°C prior to γ -radiation.

When a heat shock of 42.5°C was given prior to γ -radiation, the recovery time was about the same, but the NAD^+ level was restored to only 70% after 8 h.

Heat treatment at 44°C for 45 min prior to γ -radiation resulted in a

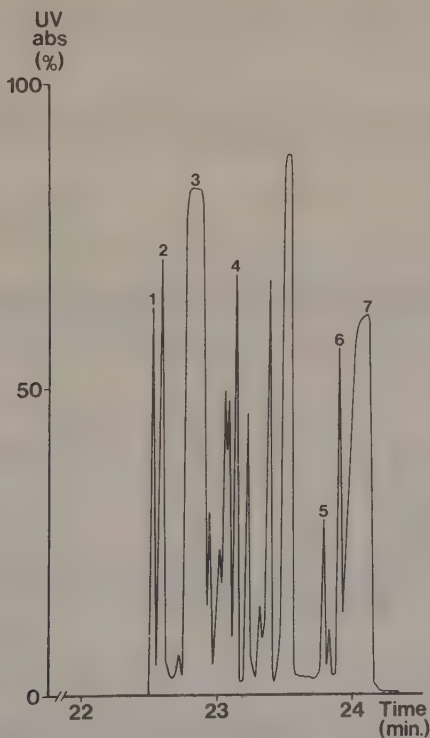


Fig. 2. Nucleotide profile from freshly isolated human mononuclear leukocytes (recorded at 254 nm). 1. UTP, 2. GTP, 3. ATP, 4. ADP, 5. AMP, 6. NAD^+ , 7. nicotinic acid (internal standard).

Table I. Adenosine nucleotide concentrations in normal freshly isolated human mononuclear leukocytes (n=18).

	Min.	Max.	Mean $\pm$ S.D
	pmol/ 10^6 cells		
ATP	897	1298	1140 $\pm$ 188
ADP	145	316	211 $\pm$ 46
AMP	58	95	69 $\pm$ 11

complete loss of recovery and the NAD^+ pool was only 20% of the original value even after 8 h of incubation.

ATP, ADP, and AMP pools after hyperthermia and γ -radiation. Data for the ATP, ADP, and AMP levels from 18 blood donors are given in Table I.

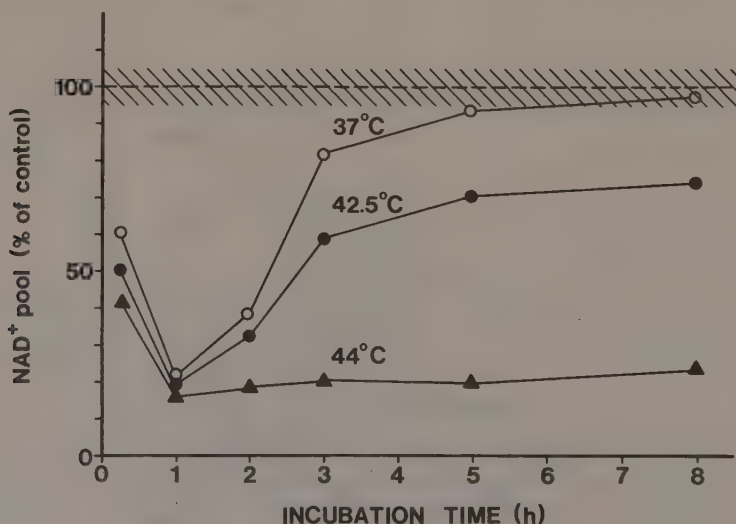


Fig. 3. NAD^+ levels relative to untreated controls versus incubation time at 37°C following hyperthermia and γ -radiation. The leukocytes were given heat treatments at 37°C (controls), 42.5°C and 44°C for 45 min prior to 30 Gy γ -radiation. The hatched area shows the range of values for unirradiated controls at 37°C, 42.5°C and 44°C.

Figures 4a-4c show the ATP levels in relative scale versus incubation time following exposure of the cells to heat alone (open circles) or given prior to 30 Gy γ -radiation (filled circles). The hyperthermia temperatures were 37°C, 42.5°C and 44°C and lasted for 45 min.

When the cells were exposed to 30 Gy γ -radiation after heat treatment, we found a reduction of ATP; the depletion being larger the higher the temperature. At lower temperatures the ATP levels began to approach those of the unirradiated samples. However, at 44°C the cells were unable to restore their ATP levels completely even after 8 h of incubation.

Figures 5a-5c show the corresponding ATP/ADP ratios in relative units versus incubation time. The ATP/ADP ratios following combined treatment

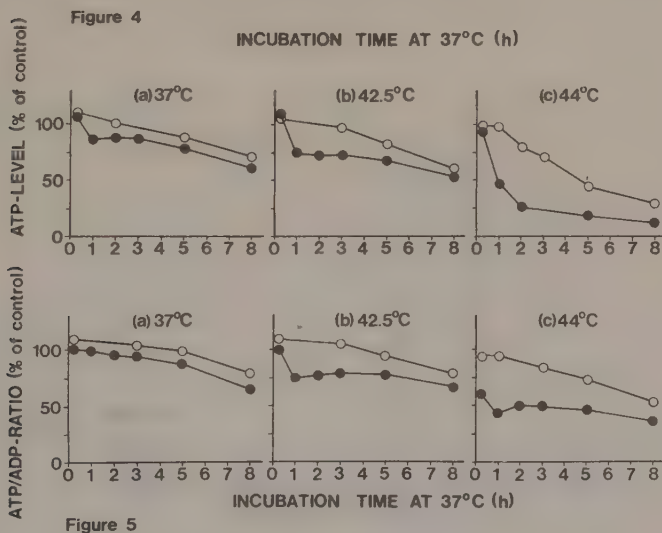


Fig. 4. ATP levels relative to untreated controls versus incubation time at 37°C. Open circles show ATP levels following hyperthermia treatment alone: (a) 37°C, (b) 42.5°C, (c) 44°C. Filled circles show ATP levels following hyperthermia at these temperatures plus 30 Gy γ -radiation prior to the heat shock.

Fig. 5. ATP/ADP ratios relative to untreated controls versus incubation time at 37°C. Symbols as in Fig. 4.

of hyperthermia and γ -radiation decreased with increasing temperature, thus reflecting an increasing lack of balance between energy utilization and energy production.

Figure 6 shows the synergistic effect of a heat shock at 44°C and 30 Gy γ -radiation on ATP, ADP, and AMP pools relative to cells exposed to hyperthermia alone. The ATP and ADP pools dropped considerably with only a slight trend toward recovery, whereas the corresponding AMP pool typically increased in response to depletion of ATP and ADP. The forms of the three curves reflect the metabolic state of energy production and utilization in the damaged cells.

The nucleoside triphosphates UTP and GTP could also be determined from the isotachophor profiles (Fig. 2) and were found to fluctuate in a manner similar to the ATP pool (data not shown).

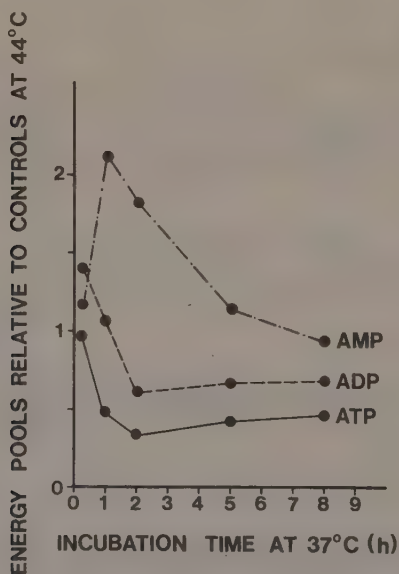


Fig. 6. ATP, ADP, and AMP levels at 44°C for 45 min 30 Gy γ -radiation relative to controls at 44°C as a function of incubation time. All data points have been divided by the values obtained after hyperthermia treatment of 44°C for 45 min only.

Effects of γ -radiation and hyperthermia on cell viability. Since mononuclear leukocytes are mainly in G0 phase of the cell cycle, then we have used trypan blue exclusion to estimate cell survival instead of other techniques that require DNA synthesis.

Figure 7 shows viability of the cells versus incubation time for the various treatments. Hyperthermia treatment at either 42.5°C or 44°C for 45 min did not in itself induce cell death within the first 10 h of incubation. This was an important control to establish that reduced pools observed over an 8 h incubation period were not due to cell necrosis induced by hyperthermic shock alone. In addition hyperthermia in combination with 30 Gy γ -radiation clearly increased the cytotoxic action of either agent administered separately (Fig. 7). This was true even in the absence of any appreciable DNA synthesis which suggests nucleotide pool imbalances directly in cell survival.

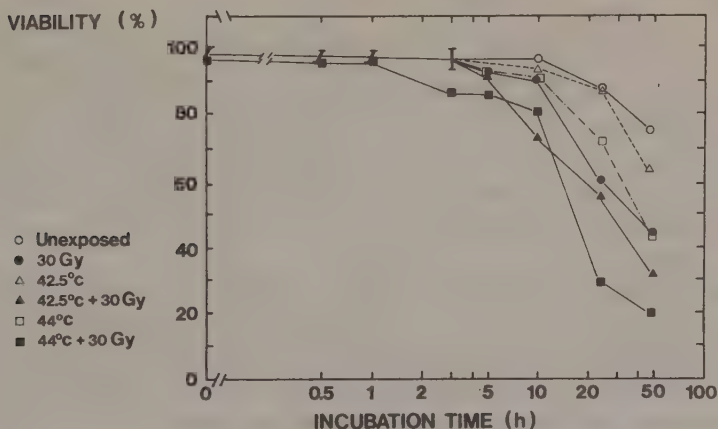


Fig. 7. Viability versus incubation time at 37°C after the various treatments. At short incubation times the viabilities were 97-100% in all treatments and those data are therefore indicated by bars.

DISCUSSION

One of the most promising molecular models proposed to explain the enhancement of cell toxicity induced by γ -radiation when combined with hyperthermic shock has centered around the hypothesis that hyperthermia inhibits the enzymatic-mediated repair of γ -ray induced DNA lesions (16-20). Recently there have been at least two lines of direct evidence in support of this hypothesis; (i) Chinese hamster ovary cellular DNA polymerase β is unusually heat sensitive (38) and (ii) *Drosophila* cellular poly(ADP-ribose)polymerase is also very heat labile (39). Our result indicate that DNA polymerase β activity, as estimated by UDS (38) and poly (ADP-ribose)polymerase activity, as estimated by NAD⁺ pool depletion (25-32), are both inhibited by hyperthermic treatment at 42.5°C for 45 min followed by γ -radiation (Figs. 1 and 3). Furthermore, we have established that hyperthermic effects on DNA repair-implicated enzymes occur in normal human mononuclear blood cells which has not previously been investigated.

The exact role of poly(ADP-ribose)polymerase in the excision repair process is not known. Both endonuclease (23) and ligase (40) are ADP-ribosylated, which, in turn, alters the capacities of these enzymes to repair DNA. It has also been reported (41, 42) that γ -radiation induces poly(ADP-ribose)polymerase activity but DNA repair of these lesions can proceed even in the presence of inhibitors for this enzyme.

In addition to DNA damaging agents, cell differentiation (43, 44) and gene expression (45) also induce DNA strand breakage and thereby activate poly(ADP-ribose)polymerase. Apparently the steady-state level of DNA strand breaks (42), which is under potential control via regulation of endonuclease- or ligase-activities by ADP-ribosylation, is crucial for involving poly(ADP-ribose)polymerase directly in the DNA repair process.

The main purpose of this study was to examine a model cell system and, at the molecular level, the effects of hyperthermia and γ -radiation in killing tumor cells in vitro. In clinical practice, high doses of γ -radiation are commonly used (i.e. > 30 Gy, ref. 1-4). This is the reason we have used such high doses of γ -radiation in this study. Under these conditions, the cells are given a vast excess of strand breaks to

repair immediately, and thus the rate limiting step is likely to be a repair enzymatic activity (e.g. ligase) which can be further activated to a higher level by ADP-ribosylation. This is supported by data regarding the cytotoxic effects of γ -radiation and poly(ADP-ribose)polymerase activity. A 0.4-1 Gy γ -radiation dose in the presence of an inhibitor of poly(ADP-ribose)polymerase was shown to reduce cell survival slightly but consistently (46). However, γ -radiation doses <0.4 Gy in the presence of the same enzyme inhibitor did not reduce cytotoxicity over the effects of γ -radiation alone (42). In this study, when 30 Gy of γ -radiation plus hyperthermia of 42.5°C for 45 min were used (i.e. conditions that prevent NAD^{+} pool regeneration), cell survival was distinctly reduced compared to that following 30 Gy γ -radiation alone (Fig. 7). In other words, when the demand for NAD^{+} is great because of excessive DNA strand breakage, then the activity of poly(ADP-ribose)polymerase becomes critical in order to increase DNA repair capacity and reduce cytotoxicity. Other explanations may also be considered.

Hyperthermia alone induces tumor regression (see e.g. ref. 47), although we have observed no effects from hyperthermia treatment alone on UDS (i.e. DNA polymerase activity, Fig. 1) or NAD^{+} pools (i.e. poly(ADP-ribose)polymerase activity, Fig. 3). On the other hand, we have shown that the energy pool balance reflected by the ATP/ADP ratios was reduced by hyperthermic treatment alone at 44°C for 45 min (Fig. 5) and other investigators (48) have shown that ATP deprivation enhances cell killing by hyperthermia alone. Hence, one hypothesis is that a depletion of ATP from hyperthermia causes a limited generation of NAD^{+} (48), which, in turn, can inhibit repair after γ -ray induced DNA damage via the reduced amount of donor substrate for the NAD^{+} consuming enzyme, poly(ADP-ribose)polymerase. This hypothesis is under investigation in our laboratory.

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EQUILIBRATION OF 3'-PHOSPHOADENOSINE-5'-PHOSPHOSULFATE (PAPS) AND UDP-N-ACETYLHEXOSAMINE WITH LABELLED PRECURSORS IN CULTURED FIBROBLASTS

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ABSTRACT

Nucleotides and sugar nucleotides were extracted from cultures of human fibroblasts with perchloric acid, separated by isotachopheresis and quantified by UV-absorption analysis at 254 nm. ATP (936 pmole/μg DNA) was as expected the dominating nucleotide pool. The energy charge was estimated to 0.9. The UDP-N-acetylhexosamine pool was also a very prominent compound (569 pmole/μg DNA).

After incubation of fibroblasts with ³H-glucosamine more than 95% of the acid soluble radioactivity was found in the UDP-N-acetylhexosamine pool. Incubation with ³⁵S-sulfate resulted in incorporation of ³⁵S-sulfate into 3'-phospho adenosine-5'-phosphosulfate (PAPS). The latter pool could, however, only be measured as radioactivity, as the amount was too small to be quantified.

Pulse labelling of fibroblasts with ³⁵S-sulfate and ³H-glucosamine from 5 min to 16 h showed that ³⁵S-PAPS was equilibrated in less than 10 min, while ³H-glucosamine required approximately 4 h to attain steady state. ¹⁴C-glucose required approximately the same time as ³H-glucosamine to reach steady state, which suggests that the reason for the long equilibration time is the slow turnover of the UDP-N-acetylhexosamine pool.

INTRODUCTION

Labelled precursors are frequently used in studies of the metabolism of various biological compounds both in vivo and in vitro. Among the factors in the selection of a precursor are its transport into the cell,

its equilibration time with intracellular precursors to attain constant specific activity and its specificity for the compound under study.

^3H -glucosamine and ^{35}S -sulfate are frequently used as precursors for studies concerning the metabolism of glycoproteins and proteoglycans (1-4). ^3H -glucosamine is readily transported into the cell through the same carrier as glucose (5). After phosphorylation by hexokinase the compound is mainly converted to UDP-N-acetylglucosamine, UDP-N-acetylglactosamine and, to a small extent, CMP-sialic acid (1, 6). ^{35}S -sulfate is rapidly transported into the cell (7, 8). Activation occurs in two steps, transsulfurylation to 3'-adenosine phosphosulfate (APS) followed by phosphorylation to PAPS (9, 10), which participate in sulfation of proteoglycans (11).

In recent studies on the incorporation of ^{35}S -sulfate and ^3H -glucosamine into proteoglycans it was detected that ^{35}S -sulfate was incorporated in a linear fashion into proteoglycans in chondrocytes (12) and fibroblasts (13) after only 10 min. ^3H -glucosamine on the other hand required considerable longer times (2-3 h) to establish linear incorporation into proteoglycans in fibroblast cultures (13). To investigate at which step this difference arises, intermediates of the sulfate and the glucosamine metabolism were isolated after pulses of ^3H -glucosamine and ^{35}S -sulfate. To achieve this a previously developed rapid extraction technique for preparing nucleotides from liver (14) had to be modified for cultures of human skin fibroblasts.

MATERIALS AND METHODS

Materials

1- ^3H -glucosamine (2.4 Ci/mmole), U- ^{14}C -glucose (0.3 Ci/mmole) and carrier free ^{35}S -sulfate were obtained from Amersham International Ltd (England). Instagel was purchased from Packard (Sweden). Nucleotides and sugar nucleotides were bought from Sigma Chemical Co (St Louis, Missouri) and mithramycin from Serva (GFR). Material for cell culture was delivered by Flow Laboratories (Hamdon, Conn.). All chemicals used were of analytical grade. PAPS were prepared using enzymes from rat liver (15).

Preparation of nucleotides.

Fibroblast cultures were established from embryonic human skin. The cells were maintained in minimum essential medium in 25 cm² plastic bottles (16). The medium was changed twice to a sulfate poor medium (17), 18 and 2 h before labelling with 2.5 μCi ³H-glucosamine and 100 μCi ³⁵S-sulfate per ml medium. In other experiments cultures were labelled with 25 μCi ¹⁴C-glucose and 2.5 μCi ³H-glucosamine per ml medium. After labelling periods ranging from 5 min to 16 h the cell layer was rinsed 3 times with 37°C phosphate buffered saline, PBS (137 mM NaCl/3 mM KCl/8 mM Na₂HPO₄/1 mM KH₂PO₄, pH=7.4) and detached by brief treatment with 0.4 % trypsin in PBS. The cells were pelleted in a bench top centrifuge for 20 s at 4500 g and +4°C. The pellet was sonicated for 30 s in a Bransonic 32 sonifierbath at +4°C in 150 μl of 0.78 M perchloric acid containing 22.5 % methanol and 0.375 μmol of nicotinic acid as internal standard. 140 μl of the supernatant was pipetted into 22 μl containing 4 μmol of triethanolamine buffered with HCl to pH=7.2, 52.5 μmol of KOH and 1.5 μg of thymol blue as indicator. The solution was adjusted to pH 7 with 2M KOH and frozen. After thawing and centrifugation the supernatant was stored at -60°C for later analysis. The whole procedure was performed at -10°C except centrifugation and sonification. The pellet containing DNA was sonicated in 250 μl of PBS containing 0.2 M Tris buffer, pH 8.0.

Analytical methods.

Isotachopheresis was performed on an LKB 2127 Tachophor (LKB, Sweden) with an electrolyte system as described previously (14). To increase separation of UV-absorbing peaks 1 μl of a spacer solution containing 0.15 mM phosphoenolpyruvic acid, 0.3 mM phosphoglyceric acid, 0.4 mM chloroacetic acid, 0.3 mM trichloroacetic acid, 0.3 mM saccharic acid, 0.4 mM glycolic acid and 0.5 mM acetic acid was run with 4 μl cell extract. Preparative isotachopheresis for radioactive determination was run in a Tachofrac kit (LKB, Sweden). No spacers were used when the equilibration of PAPS and UDP-N-acetylhexosamine was studied with ³⁵S-sulfate and ³H-glucosamine as precursors. In experiments with ¹⁴C-glucose and ³H-glucosamine, 1.5 mM of citric acid and 1.5 mM of glycolic acid were added to separate UDP-N-acetylhexosamine from the broad spectra of labelled intermediates metabolized from ¹⁴C-glucose. The cellulose acetate strip with the fractionated sample was treated

as described previously (18).

Paper chromatography was performed on Whatman 3 MM paper for 16 h in solvent A: ethanol - 1 M NH_4Ac , pH7.0 (7:3 vol/vol) or 60 h in solvent B: butanol - pyridin - 0.1 M HCl (5:3:2 vol/vol).

DNA analyses were performed with the mithramycin method (19), the diphenylamine method (20) and the bisbenzimidazole method (21).

Radioactivity was measured in a Packard 300 C with automatic quench correction.

RESULTS

A typical separation of nucleotides and sugar nucleotides from fibroblasts by isotachopheresis is shown in Fig. 1. The concentration of some of the nucleotides is given in Table 1. The relation of ATP, ADP and AMP gives an energy charge of 0.9. The ratio of UTP/UMP indicates minimal UDP-sugar break down. Two sugar nucleotides, UDP-glucuronic acid and UDP-N-acetylhexosamine, are obtained free from other UV-absorbing compounds and in concentrations high enough to quantitate. The UDP-N-acetylhexosamine pool is the largest of the sugar nucleotides in skin fibroblasts (569 pmole/ μg DNA) and only the concentration of ATP is larger.

Table I. Concentration of various nucleotides and sugar nucleotides in fibroblasts Fibroblasts in culture were extracted as described in the experimental section. After neutralization nucleotides were quantified by isotachopheresis. The figures are given as mean $\pm$ s.e.m. (n=6).

Nucleotide	pmole/ μg DNA
UTP	186 $\pm$ 5.2
GTP	211 $\pm$ 5.7
UDP-glucuronic acid	40 $\pm$ 2.0
ATP	936 $\pm$ 12.0
UDP-N-acetylhexosamine	569 $\pm$ 35.6
ADP	122 $\pm$ 13.1
UMP	<15
AMP	19 $\pm$ 5.1
NAD	213 $\pm$ 7.3

UDP-glucose which is obtained in the same peak as NADPH (Fig. 1.) is found in lower concentration than the UDP-N-acetylhexosamine. Added PAPS migrates between UDP-glucuronic acid and ATP. No trace of this compound can, however, be detected in the fibroblasts (Fig. 1.) which indicates that skin fibroblasts contain less than 5 pmole PAPS/ μ g of DNA.

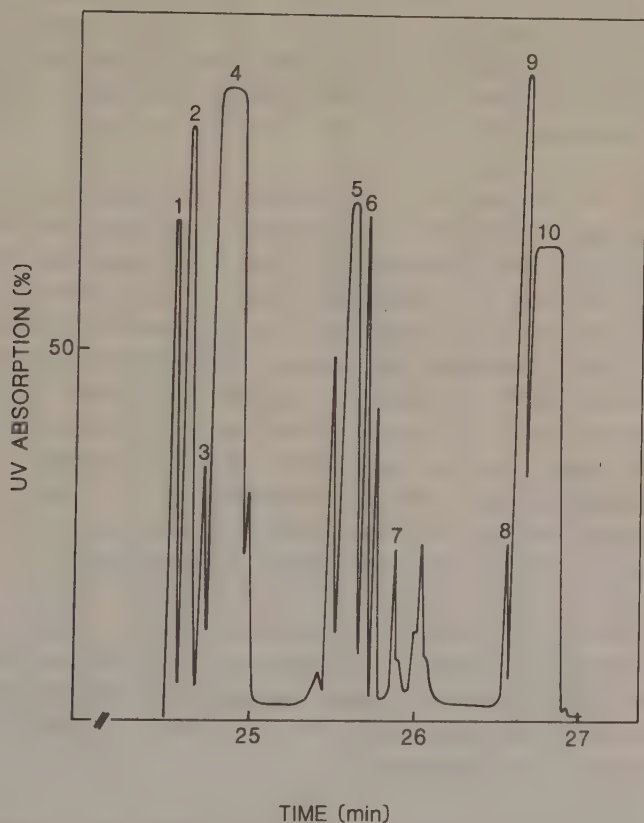


Fig. 1. Separation of perchloric acid extract from fibroblasts by isotachopheresis: The extract was prepared from cultured fibroblasts and spacers were added to the extract before isotachopheresis as described in the experimental section. The quantified UV-absorbing peaks are 1; UTP, 2; GTP, 3; UDP-glucuronic acid, 4; ATP, 5; UDP-N-acetylhexosamine, 6; ADP, 7; UMP, 8; AMP, 9; NAD, 10; internal standard (nicotinic acid). For identification of other peaks see reference (14). Absorption is defined as 100% - transmittance.

In preliminary experiments 25 μCi of ^3H -glucosamine or 250 μCi of ^{35}S -sulfate/ml medium did not change the nucleotide pattern compared to controls. After incorporation of ^{35}S -sulfate (100 $\mu\text{Ci}/\text{ml}$) for 8 h only one peak of ^{35}S -radioactivity was obtained on the cellulose acetate strip after preparative isotachopheresis of the nucleotide extract (Fig. 2.). This peak migrated in the same position as standard PAPS. Further identification was afforded by paper chromatography in solvent A (data not shown). In addition to the ^{35}S -PAPS peak a large peak of free ^{35}S -sulfate was obtained on the chromatogram amounting to approximately 92% of the total ^{35}S -sulfate activity.

After the incorporation of ^3H -glucosamine (2.5 $\mu\text{Ci}/\text{ml}$) into fibroblasts for 8 h, preparative isotachopheresis of the nucleotide extract revealed one major peak migrating as UDP-N-acetylhexosamine and one minor peak (Fig. 2.). Paper chromatography of the extract in solvent A gave approximately the same result: one major peak chromatographing as UDP-N-acetylglucosamine with one minor peak ahead of it. In the isotachopheretic system the small peak was approximately 4% of the total ^3H -radioactivity. This minor component may contain CMP-sialic acid and/or N-acetylglucosaminephosphate. Furthermore the recovery of radioactive material after isotachopheresis was 100 %, which together with data from the paper chromatogram, indicates that only minute amounts of free glucosamine is present intracellularly.

The material migrating as UDP-N-acetylhexosamine on paper chromatography was eluted and subjected to hydrolysis and then rechromatographed on paper in solvent B. Galactosamine and glucosamine were the only radioactive components detected and approximately 29% of the total ^3H -radioactivity was galactosamine.

Pulse-labelling from 5 min to 8 h of fibroblasts with ^{35}S -sulfate and ^3H -glucosamine resulted in increasing incorporation into PAPS and UDP-N-acetylhexosamine respectively (Fig. 3.). In all experiments performed ^{35}S -sulfate transport into the cell and formation of ^{35}S -PAPS reached a steady state within 10 min (Fig. 3.). After all pulses the proportion of ^{35}S -PAPS to free ^{35}S -sulfate was the same.

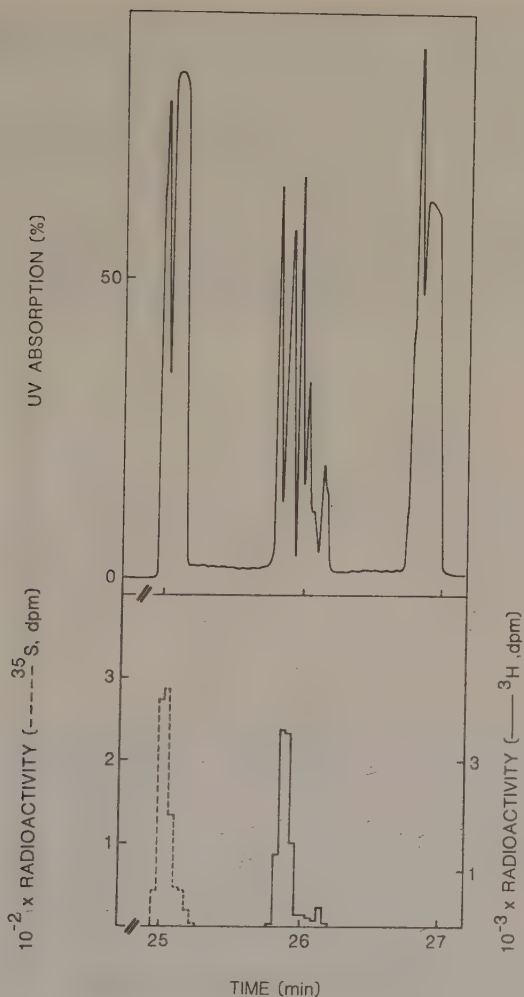


Fig. 2. Fractionation of an extract from ^3H -glucosamine and ^{35}S -sulfate labelled fibroblast culture by isotachopheresis: An extract was prepared from a fibroblast culture which had been labelled for 8 h with ^3H -glucosamine and ^{35}S -sulfate as described in the experimental section. The extract was fractionated by isotachopheresis. After monitoring the separation at 254 nm, the material was transferred to a moving cellulose acetate strip, which was cut and solubilized in acetic acid and counted for radioactivity. a) Detection was performed at 254 nm. b) Radioactivity: - - - - ^{35}S , — ^3H .

The equilibration of ^3H -glucosamine with UDP-N-acetylhexosamine pool required, however, considerable longer times. In experiments with some fibroblast lines at least 4 h of incubation was required before the UDP-N-acetylhexosamine pool attained steady state (Fig. 4.). Neither the proportion of the tentative N-acetylglucosaminophosphate/CMP-sialic acid compounds to UDP-N-acetylhexosamine (4 %) nor the proportion of galactosamine (29 %) to total hexosamine of the UDP-N-acetylhexosamine pool changed during the experiments. The amount of UDP-N-acetylhexosamine in nmole/ μg DNA did not change during the pulse experiment. Accordingly the specific activity of the UDP-N-acetylhexosamine pool increased for approximately 4 h (Fig. 4.).

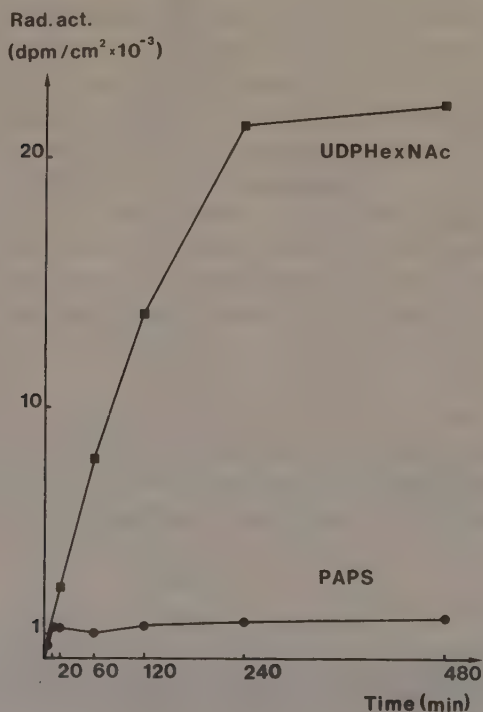


Fig. 3. Equilibration of ^{35}S -sulfate with PAPS (—●—●—) and ^3H -glucosamine with UDP-N-acetylhexosamine (—■—■—): After pulse labelling of the fibroblasts nucleotides were prepared as described in the experimental section and separated as in Fig. 2.

In some experiments ^{14}C -glucose and ^3H -glucosamine was administered to the cell cultures simultaneously in order to compare their respective equilibration time with the UDP-N-acetylhexosamine pool. It is evident from Fig. 5. that the two precursors were incorporated into the UDP-N-acetylhexosamine at the same relative rate. The ratio of $^3\text{H}/^{14}\text{C}$ of the UDP-N-acetylhexosamine pool was constant during the experiment (Fig. 5.). Using the specific activities of the radioactive precursors of the medium it can be calculated that the amount of glucose which is channelled through the UDP-N-acetylhexosamine pool is approximately 200 fold larger than that of glucosamine.

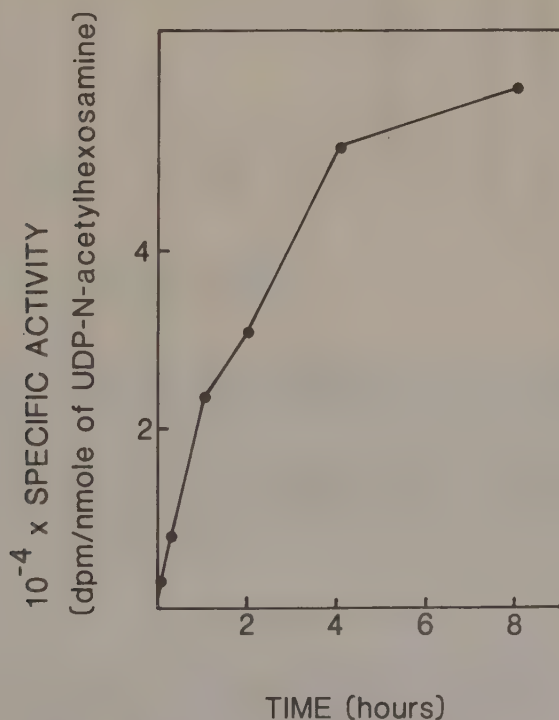


Fig. 4. Increase of specific activity of the UDP-N-acetyl-hexosamine pool: The specific activity was determined using levels of nmole UDP-N-acetylhexosamine and dpm obtained by the tachophrac version of isotachopheresis (Fig. 2).

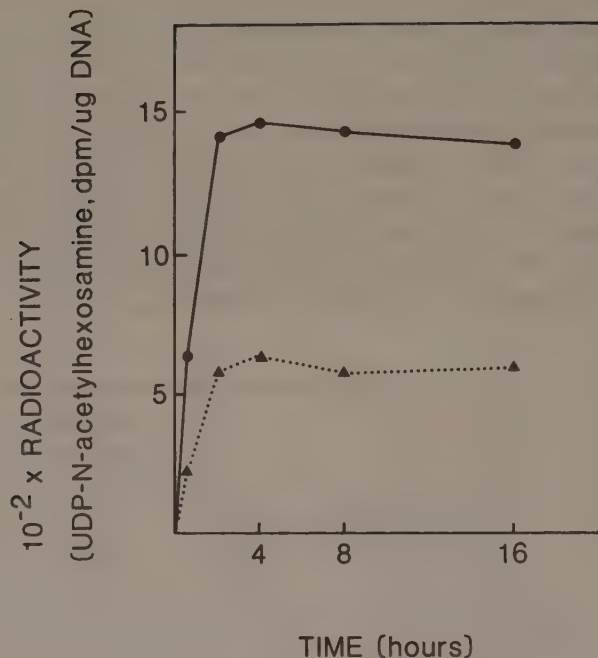


Fig. 5. Equilibration of ^3H -glucosamine (—●—●—) and ^{14}C -glucose (···▲···▲···) with the UDP-N-acetyl hexosamine pool: After pulse labelling the cells were extracted and spacers were added to the extract to achieve a good separation of radioactive UDP-N-acetylhexosamine from other radioactive compounds, as described in the experimental section. The cells used in this experiment had a shorter equilibration time. This is probably due to the use of a cell line in a lower passage.

DISCUSSION

Studies of nucleotides and sugar nucleotides are hampered by the diversity of these groups and their lability at high and low pH levels. The described procedure enables us to quantitate several nucleotides and two sugar nucleotides with isotachopheresis in less than 30 min. The size of ATP, ADP and AMP pools and the energy charge of 0.9 indicates that the cells are in good condition and that the extraction procedure is adequate. The low concentration of UMP indicates that the UTP and UDP sugar

pools are unchanged after the preparatory procedure. The data are in agreement with those of Nissinen (22) using high pressure liquid chromatography, except for the amount of NAD^+ which is underestimated and UDP-N-acetylhexosamine which is not identified. The glycoconjugate precursors, UDP-glucuronic acid and UDP-N-acetylhexosamine, can be quantitated using isotachopheresis. It is noteworthy that the latter pool is one of the most prominent of the nucleotide pools of fibroblasts. Unfortunately UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine cannot be separated by the above procedure. UDP-hexoses are not separated from NADPH. PAPS is present in too low a concentration to be quantified. Improvement of the isotachopheresis technique by introducing new spacers and using a column coupling system (23, 24) to increase the sample may be possible.

The transport of ^{35}S -sulfate into the cell is ascribed to a carrier mediated process (7, 8) and it seems to be rapid in fibroblasts. Further equilibration of intracellular ^{35}S -sulfate with PAPS is also a rapid process since the ratio of ^{35}S -sulfate to ^{35}S -PAPS is constant from 5 min up to 8 h. The ratio (92/8) may, however, be wrong as ^{35}S -sulfate can be exchanged with the surrounding medium during trypsinization and centrifugation, a process requiring 3 min. Anyhow since equilibrium with PAPS is attained in less than 10 min ^{35}S -sulfate is a useful precursor for studies on biosynthesis of sulfated compounds.

Glucosamine is transported into the cell by the same carrier as glucose (5, 25) and then phosphorylated to glucosamine-6-phosphate by hexokinase (1). After acetylation of the amino group and transfer of the phosphate group to carbon-1 it is converted to the UDP-N-acetylglucosamine. The UDP-N-acetylhexosamine pool is large and more than 95% of the radioactivity is found in this pool, which is in agreement with earlier work (6, 26). The remaining radioactivity originates most likely from N-acetylglucosamine-phosphate and/or CMP-sialic acid. As a tracer dose of glucosamine was used in the experiment, no UDP-glucosamine could be seen as when higher doses are used (6, 26). A tracer dose of ^3H -glucosamine resulted in a long equilibration time (2-4 h) to achieve constant specific activity of the UDP-N-acetylhexosamine pool (Fig. 4). The shorter time was noted in an experiment performed on another fibroblast cell

line which, furthermore, was in a low passage. The reason for the long equilibration time may be a slow transport of glucosamine over the cell membrane in the presence of the vast excess of glucose in the medium as these sugars apparently use the same carrier (5, 25). This does not seem to be the case, however, as ^{14}C -glucose seems to require the same long equilibration time (Fig. 5). Furthermore no build-up of intermediates on the route to UDP-N-acetylhexosamine was noted, which indicates that the rate limiting step are early. Kornfeld et al (27) showed that the first reaction, from fructose-6-phosphate to glucosamine-6-phosphate, was subjected to feed-back regulation of UDP-N-acetylglucosamine. This step is, however, circumvented when glucosamine is used as precursor. It is therefore likely that the large pool size of the UDP-N-acetylhexosamine in combination with a moderate turnover results in a long equilibration time. In conclusion, it should be pointed out that studies with labelled precursors must be carried out under carefully controlled conditions regarding specific activities of the intermediates, if valid interpretations of experiments are to be made.

ABBREVIATIONS

PAPS 3'-phosphoadenosine-5'-phosphosulfate
 PBS phosphate buffered saline (137 mM NaCl/3 mM KCl/8mM Na_2HPO_4 /
 1 mM KH_2PO_4 , pH=7.4)

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